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P012842GB AMD

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Patents ADP number (if you know it)

If the applicant is a corporate body, give the country/state of its incorporation

United Kingdom

RE 147 19.3.03

4. Title of the invention

Compounds

5. Name of your agent (if you have one)

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Country

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11.

I/We request the grant of a patent on the basis of this application.

Signature *Anna Denholm* Date 19 April 2002
D Young & Co (Agents for the Applicants)

12. Name and daytime telephone number of person to contact in the United Kingdom

Anna Denholm

023 8071 9500

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Compounds

The present invention relates to new anionic glycodendrimers having new biological activity, processes for preparing them and their use in medicine including veterinary
5 medicine.

Dendrimers are a class of polymeric compounds that can be distinguished from conventional linear polymers by their highly branched, but circular and symmetrical architecture (Figure 1a and Figure 1b). They have a molecular structure that can be much
10 more precisely defined than is possible for linear polymers (Tomalia DA, Naylor AM, Goddard III WA. (1990) Starburst dendrimers: molecular level control of size, shape, surface chemistry, topology and flexibility from atoms to macroscopic matter. *Angewandte Chemie-International Edition in English* **29**, 138-175). STARBURST™ polymers (a trade mark of Dendritech Inc. Midland, Michigan) are dense star polymers.
15 They are a particular type of dendrimer which is formed by the generational addition of branched layers from a core.

WO 95/24221 [Dow Chemical Company] discloses dendritic polymer conjugates comprising dense star polymers complexed or conjugated with biological response
20 modifiers.

The reactive end groups of dendrimers are present at the periphery of the molecule and these groups can be cationic or anionic (Newkome GR, Nayak A, Behera RK, Moorefield CN, Baker GR & Johnson AL. (1992) Chemistry of micelles series .22. cascade polymers - synthesis and characterization of 4-directional spherical dendritic macromolecules based
25 on adamantane. *Journal of Organic Chemistry* **57**, 358-362). End group modification is usually employed in dendrimers that have been prepared by the divergent approach:- i.e. poly(amidoamine) dendrimers (PAMAM dendrimers) and poly(propyleneimine) dendrimers (DAB dendrimers). Such modification of the functional groups on the outer
30 surface of the dendrimer can significantly alter the biological properties of these molecules as a consequence of co-operative effects (Jansen JFGA, de Brabander-van de Berg EMM & Meijer EW. (1994) Encapsulation of guest molecules into a dendritic box. *Science* **266**, 1226-1229). Compounds able to produce co-operative effects are very important in modulating biological control mechanisms in the immune system.

Previously it has not proved possible to covalently couple biological molecules to anionic dendrimers in order to make new compounds with novel and unique biological activity.

- 5 The present invention now provides a process to covalently link biologically active molecules to anionic dendrimers wherein the dendrimers are reacted with the biologically active molecule in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Figure 2). The biologically active agents include drugs, nucleic acids (in particular DNA), proteins, peptides, carbohydrates and antibodies of natural or synthetic 10 origin. The process of the present invention has the advantages of comprising single step, water based chemistry, which can be performed at room temperature. The process of the present invention has the further advantage that it avoids the need for organic solvents that are often toxic *in vivo*. Furthermore, organic solvents require additional, complicated and expensive purification of the product from the organic solvent. Conjugation in an 15 aqueous environment facilitates the purification of the final product. This is important from an industrial, manufacturing perspective for the new drugs. A particular advantage of the process of the present invention is that conjugation can be carried out at room temperature.
- 20 The process of the present invention is particularly useful for the preparation of anionic glycodendrimers.

In 1996, Roy defined glycodendrimers as "a new class of low molecular weight multiantennary carbohydrate containing biopolymers" (Pagé D & Roy R. (1997) 25 Synthesis and biological properties of mannosylated Starburst poly(amidoamine) dendrimers. *Bioconjugate Chemistry* 8, 714-723). They were synthesised by convergent approaches with incorporation of various carbohydrate structures at the last step.

Glycodendrimers have been described by the manner in which saccharide residues are 30 incorporated as complexes or conjugates into them as one of two types (Ashton PR, Boyd SE, Brown CL, Nepogodiev SA, Meijer EW, Peerlings HWI & Stoddart JF. (1997) Synthesis of glycodendrimers by modification of poly(propyleneimine) dendrimers. *Chemistry a European Journal* 3 (6), 974-98420):

- (i) carbohydrate coated dendrimers characterised by the presence of saccharide residues attached to the end groups of the dendrimer.
- (ii) fully carbohydrate dendrimers where the saccharides are used as multifunctional building blocks giving rise to dendrimers that are totally carbohydrate.

5

Although it is known that neoglycoconjugates can be prepared by linking saccharides to carriers, only those pre-formed dendrimers bearing primary amino groups on their periphery have been investigated as a macromolecular support for the attachment of carbohydrates (Ashton PR, Boyd SE, Brown CL, Nepogodiev SA, Meijer EW, Peerlings HWI & Stoddart JF. (1997) Synthesis of glycodendrimers by modification of poly(propyleneimine) dendrimers. Chemistry a European Journal 3 (6), 974-984). In these cases, the primary amine terminated dendrimers can be modified with relative ease with a variety of reagents. In contrast, it had not been possible to modify the carboxylic acid groups on the surface of carboxylic terminated, anionic dendrimers in order to give the dendrimer different functional activity. The biological problem that this has led to is that aminic terminated dendrimers are considerably more toxic than the carboxylic terminated dendrimers *in vivo* (Malik N, Wiwattanapatapee R, Klopsch R, Lorenz K, Frey H, Weener JW, Meijer EW, Paulus W & Duncan R. (2000) Dendrimers: Relationship between structure and biocompatibility *in vitro*, and preliminary studies on the 15 biodistribution of ¹²⁵I-labelled polyamidoamine dendrimers *in vivo*. Journal of Controlled Release 65, 133-148). Therefore it was important to develop new glycodendrimers that 20 were based upon carboxylic terminated dendrimers as the starting material.

In a further aspect, the present invention now provides new anionic glycodendrimers 25 comprising covalently linked carboxylic terminated dendrimers. Preferably the dendrimers are PAMAM dendrimers, particularly preferred PAMAM dendrimers are generation 3.5 (Generation is a term which relates to the way the molecules are synthesised and their molecular weight.) Preferably, the dendrimers are covalently linked to compounds containing aminic groups such as aminic sugars and sulphated 30 sugars. Preferred sugars are glucosamine or sulphated glucosamine.

The one-step reaction can also be used for the covalent conjugation of PAMAM anionic dendrimers gen 2.5 to sugars containing aminic groups and to sulfated sugars containing aminic groups. Linear molecules, that is molecules without a ring structure, can also be

attached to the anionic PAMAM dendrimers using the same chemistry. This has been successfully achieved for 3-amino-1-propanesulfonic acid for example. Typically the covalent linker bond is stable over a period of at least 90 days.

- 5 Subsequent purification of the products has also been made simple and straightforward. Until now, it has only been possible to covalently link molecules to cationic PAMAM dendrimers using multistep organic chemistry followed by a large number of purification steps.
- 10 Carboxylic acid terminated anionic PAMAM dendrimers have a remarkable lack of toxicity both *in vitro* and *in vivo* compared to cationic PAMAM dendrimers (Tsutsumiuchi K, Aoi K, & Okada M. (1999) Globular carbohydrate macromolecule "Sugar Balls" IV. Synthesis of dendritic nanocapsules with molecular recognition sites on periphery. *Polymer Journal* **31**, 935-941). Cationic dendrimers cause red cell haemolysis at a concentration of 10 µg/ml whilst anionic dendrimers have no such effect even at 2,000 µg/ml. Anionic dendrimers have a longer circulation time in the systemic vascular circulation than cationic dendrimers, and this is associated with a lower degree of accumulation of the molecule in the liver. This inherent toxicity of higher generation cationic dendrimers means that they are unlikely to be suitable or safe for either high dose 15 intravenous administration or for repeated intravenous administration (Malik N, Wiwattanapatapee R, Klopsch R, Lorenz K, Frey H, Weener JW, Meijer EW, Paulus W & Duncan R. (2000) Dendrimers: Relationship between structure and biocompatibility *in vitro*, and preliminary studies on the biodistribution of ¹²⁵I-labelled polyamidoamine 20 dendrimers *in vivo*. *Journal of Controlled Release* **65**, 133-148).
- 25 In a preferred aspect of the present invention the attachment of non-biologically active molecules to anionic PAMAM dendrimers allows the effective manipulation of chemokines and, in turn cytokines, as well as new blood vessel formation even though 30 dendrimers, and molecules such as glucosamine and glucosamine 6-sulphate have no intrinsic anti-inflammatory or antiangiogenic activity. Glucosamine is also a sugar moiety that can be conjugated to the dendrimer core via the ability of the amine group to react with the carboxylate groups present on the surface of anionic dendrimers. This leads to the formation of a stable amide bond. Glucosamine containing various combinations of

sulfate moieties at the 3', 4' and 6' positions of the glucan moiety were also suitable for attachment using the same chemistry. This resulted in the formation of a new anti-angiogenic glycodendrimer with immunomodulatory properties and antiangiogenic properties.

5 The number of molecules of sugar or sulfated sugar covalently bound to the surface of the dendrimer increases in proportion to the number of carboxylic groups that are present on the surface of the dendrimer. The amount of glucosamine linked to the dendrimers, expressed as a percentage of converted carboxylic groups ranges from 1.5 to 10.1 %. In 10 the case of the sulfated dendrimers, the sulphur content increased in the following order: monosulfated sugar (2.79 % m:m S); disulfated sugar (5.78 % m:m S); trisulfated sugar (7.93 % m:m S). The amount of sulfate present in the conjugate was 6 % for glucosamine 6-sulfate, 11 % for glucosamine 3,6-disulfate, and 15 % for glucosamine 3,4,6-trisulfate.

15 The present invention also provides for the use of the compounds of the present invention in medicine.

These new anionic glycodendrimers downregulate the immune system by acting first and foremost on the release of chemokines. The compounds inhibit the release of pro-inflammation 20 chemokines which, in turn, inhibits the release of pro-inflammatory cytokines. The compounds of the invention also have antiangiogenic properties.

Accordingly, the present invention also provides for the use of the compounds in the manufacture of a medicament for the treatment of those diseases where chemokines and 25 cytokines are increased.

The compounds of the invention are particularly useful in the treatment of sepsis, rheumatological diseases and angiogenesis in animals and man.

30 Particular rheumatological conditions include rheumatoid arthritis, juvenile chronic arthritis, psoriatic arthritis, reactive arthritis occurring after an infection, active ankylosing spondylitis, arthritis associated with inflammatory bowel disease, Behcet's disease including Behcet's disease with panuveitis and/or retinal vasculitis, psoriasis, inflammatory bowel disease (Crohn's disease, ulcerative colitis), allogeneic organ

transplantation, graft versus host disease, diseases associated with metastatic tumour cell growth.

5 The sepsis syndrome refers to sepsis plus impaired organ perfusion. The spectrum of clinical syndromes ranging from bacteremia to sepsis to severe sepsis to septic shock to refractory septic shock and to the systemic inflammatory response syndrome represents a continuum in which localised inflammation is at one end with a severe generalised inflammatory response leading to multi-organ failure being at the other end of the spectrum.

10 In a further aspect, the present invention provides pharmaceutical formulations comprising glycodendrimers of the invention optionally plus a carrier.

15 The compounds and formulations of the invention are suitable for administration intravenously, orally, intraperitoneally, topically (skin), buccal, rectally, to the surface of the skin, transdermal (slow release preparation), subcutaneously, intramuscularly, intranasally, by aerosol, pulmonary, and directly to the eye.

20 Particularly preferred compounds according to the invention are dendrimer gen. 3.5-glucosamine and dendrimer gen 3.5-glucosamine 6-sulphate which have been proven to be useful.

25 For example in human cell lines, the dendrimer gen 3.5-glucosamine showed IC_{50} toxicity values that ranged from 1,825 to 3,000 μ g/ml, and the dendrimer gen 3.5-glucosamine 6-sulfate showed IC_{50} toxicity values that ranged from 246 –307 μ g/ml. In freshly isolated, human peripheral blood mononuclear (PBMN) cells and in freshly isolated human monocyte-derived-macrophages (MDM), the IC_{50} toxicity value for dendrimer gen 3.5-glucosamine was 340-360 μ g/ml. In freshly isolated, human peripheral blood mononuclear cells and in freshly isolated human monocyte-derived macrophages, the IC_{50} toxicity value for dendrimer gen 3.5-glucosamine 6-sulfate, the dendrimer gen 3.5-glucosamine 3,6-disulfate, and the dendrimer gen 3.5-glucosamine 3,4,6-trisulfate was 230-260 μ g/ml.

Single donor MDMs were cultured with dendrimer gen 3.5-glucosamine up to a concentration of 150 μ g/ml for 72 h. No reduction in cell viability was seen. Single donor HUVECs were cultured with dendrimer gen 3.5-glucosamine up to a concentration of 100 μ g/ml for 72 h. No reduction in cell viability was seen. The dendrimer gen 3.5-glucosamine was not toxic to Wistar rats when injected intravenously at a concentration of up to 30 mg/kg.

Neither dendrimer gen 3.5-glucosamine nor dendrimer gen 3.5-glucosamine 6-sulfate affected the release of chemokines or cytokines from immune cells that were in their normal resting state. Activity of these two compounds was only seen when these compounds were cultured with cells that had been immunologically stimulated by an antigen in some way. Therefore, these two compounds do not interfere with the normal homeostatic control under which primary, human cells operate on a day-to-day basis. As such, they differ from all other sulphated polysaccharides.

When single donor PBMN cells are cultured with dendrimer gen 3.5-glucosamine at 100 μ g/ml the release of macrophage inflammatory protein-1 α (MIP-1 α ; chemokine), macrophage inflammatory protein-1 β (MIP-1 β ; chemokine), Interleukin-8 (IL-8; chemokine), Tumor Necrosis Factor- α (TNF- α ; cytokine), Interleukin-1 β (IL-1 β ; cytokine) and Interleukin-6 (IL-6; cytokine) by lipopolysaccharide (LPS) at 5 ng/ml is significantly reduced. When single donor PBMN cells are cultured with dendrimer gen 3.5-glucosamine at 200 μ g/ml the release of MIP-1 α , MIP-1 β , IL-8, TNF- α , IL-1 β and IL-6 by LPS at 5 ng/ml is significantly reduced. When single donor PBMN cells are cultured with LPS at 5 ng/ml for 30 min, or 1 hour, or 2 hours, or 3 hours, or 4 hours before the addition of dendrimer gen 3.5-glucosamine at 100 μ g/ml, the release of MIP-1 α , MIP-1 β , IL-8, TNF- α , IL-1 β and IL-6 is significantly reduced. It has been shown that the dendrimer gen 3.5-glucosamine is not mediating its activity by binding to the LPS used in these experiments. It is believed that the dendrimer gen. 3.5-glucosamine and dendrimer gen. 3.5-glucosamine 6-sulphate mediate their effects by acting on toll-like receptors on the cell surface.

When single donor PBMN cells are cultured with dendrimer gen 3.5-glucosamine at 300 μ g/ml whose loading with glucosamine is only 3% (rather than the 7% used in all other

experiments), the release of MIP-1 α , MIP-1 β , IL-8, TNF- α , IL-1 β and IL-6 by LPS at 5 ng/ml is significantly reduced. When single donor PBMN cells from 2 or 3 individuals are mixed together and dendrimer gen 3.5-glucosamine is added at 50 μ g/ml, there is a significant reduction in the release of MIP-1 β and TNF- α . When single donor PBMN cells from 2 or 3 individuals are mixed together and dendrimer gen 3.5-glucosamine is added at 100 μ g/ml, followed by LPS at 5 ng/ml or 10 ng/ml, there is a significant reduction in the release of MIP-1 β .

None of the compounds described (ie: dendrimer gen 3.5-glucosamine, dendrimer gen 3.5-glucosamine 6-sulfate, dendrimer gen 3.5-glucosamine 3,6-disulfate, or dendrimer gen 3.5-glucosamine 3,4,6-trisulfate) has any anticoagulant activity as determined using the following in vitro assays:- kaolin partial thromboplastin time, prothrombin time, thrombin time and a Factor Xa assay. No anticoagulant activity was seen at the maximum concentration of 200 μ g/ml that was analysed for all of the compounds tested. For dendrimer gen 3.5-glucosamine, this was also confirmed at a concentration of 300 μ g/ml. The compounds of the present invention constitute the first group of sulphated polysaccharides that have been synthesised that have no anticoagulant activity. They are therefore particularly suitable for intravenous administration in animal and in man.

New vessel formation is inhibited by a direct effect of dendrimer gen 3.5-glucosamine 6-sulfate on normal endothelial cells. Its effect is to prevent endothelial cells coming together to form new endothelial vessels and to form new blood vessels. In contrast to all other sulphated polysaccharides that have been developed as anti-angiogenic compounds, the dendrimer gen 3.5-glucosamine 6-sulfate synthesised has no anticoagulant activity. The dendrimer gen 3.5-glucosamine 6-sulfate does not affect cell viability or the growth characteristics of PBMN cells or MDMs when present at a concentration of up to 150 μ g/ml in cultures of these cells that are maintained for up to 5 days.

The dendrimer gen 3.5-glucosamine 6-sulfate construct is not toxic to HUVECs when added to cultures of these cells up to a concentration of 100 μ g/ml for up to 72 hours.

When single donor PBMN cells are cultured with dendrimer gen 3.5-glucosamine 6-sulfate at 150 μ g/ml or at 200 μ g/ml, the release of MIP-1 β and TNF- α by LPS at 5 ng/ml is significantly reduced.

5 When single donor MDMs from 4 individuals are mixed and dendrimer gen 3.5-glucosamine or dendrimer gen 3.5-glucosamine 6-sulfate is added at 25 μ g/ml, there is a significant reduction in the release of MIP-1 β .

10 Using the human umbilical vein endothelial cell Matrigel assay as an *in vitro* model of angiogenesis, dendrimer gen 3.5-glucosamine 6-sulfate inhibits new vessel formation and prevents new blood vessel formation; ie: angiogenesis.

15 Using an *in vitro* angiogenesis assay based on the outgrowth of new blood vessels from human placentas, dendrimer gen 3.5-glucosamine 6-sulfate causes a reduction in the number of new vessels that are formed when it is present at 25 μ g/ml. The effect is significantly greater at 50 μ g/ml.

20 The compounds of the invention are suitable for reducing the severity of the sepsis syndrome in animals and in man. They can be administered intravenously, orally or intraperitoneally at a concentration ranging from 2.5 to 2,500 μ g/ml. The preferred concentration is 25 μ g/ml to 250 μ g/ml.

25 In rheumatoid arthritis the compounds of the invention slow, abort or even prevent the acute-on-chronic tissue damage that is seen in this disease. The administration of the compounds in rheumatoid arthritis (especially early on in the course of the disease) would significantly reduce the release of the pro-inflammatory chemokines (MIP-1 α , MIP-1 β and IL-8) which, in turn, would reduce or abolish the release of the pro-inflammatory cytokines (TNF- α , IL-1 and IL-6).

30 The simultaneous administration of mixtures of two or more compounds of the invention to inhibit the release of pro-inflammatory chemokines and, in turn, inhibit the release of pro-inflammatory cytokines, and to concurrently inhibit angiogenesis is able to result in considerable synergy of action with minimal toxicity. For example the simultaneous

administration of dendrimer gen 3.5-glucosamine and dendrimer gen 3.5-glucosamine 6-sulfate provides considerable synergy of action with lower doses and less frequent administration being possible.

- 5 The application of topical (ie: skin) preparations of the compounds of the invention to the active plaque lesions of psoriasis (especially early on in the course of the disease) results in the healing of these lesions by reducing or preventing endothelial cell proliferation and the angiogenic response that is driving the development of the psoriatic lesion.
- 10 The application of compounds of the invention to reduce or prevent new endothelial cell formation into vessels during its earliest stages is beneficial for the treatment of the skin lesions of psoriasis. In particular the administration of compounds such as dendrimer gen 3.5-glucosamine is suitable to reduce the release of the pro-inflammatory chemokines (MIP-1 α , MIP-1 β and IL-8) and, in turn, the release of the pro-inflammatory cytokines (TNF- α , IL-1 and IL-6) in psoriasis.
- 15

The simultaneous administration of mixtures such as dendrimer glucosamine and dendrimer glucosamine 6-sulfate to inhibit angiogenesis and to inhibit the release of pro-inflammatory chemokines and, in turn, the release of the pro-inflammatory cytokines results in considerable synergy of action with minimal toxicity in psoriasis. In particular, this provides a therapeutic benefit to the skin lesions of psoriasis as well as to the rheumatological manifestations of psoriatic arthropathy.

- 20
- 25 The compounds of the invention are suitable for administration intravenously, orally, buccal, intraperitoneally, into the joint space, rectally, to the surface of the skin, transdermal (slow release preparation), subcutaneously, intramuscularly, intranasally, to the eyes as eye drops, by intravitreal (i.e. into the eye) injection, or by aerosol (eg. topically to the lungs) at a concentration ranging from 2.5 to 2,500 μ g/ml. The preferred concentration is 25 μ g/ml to 250 μ g/ml.

30 Crohn's Disease and Ulcerative Colitis are the major forms of inflammatory bowel disease. The oral or intravenous administration of compounds such as dendrimer gen 3.5-glucosamine and dendrimer gen 3.5-glucosamine 6-sulfate are especially suitable early on

in the course of the disease. The drug would accumulate in the tissues associated with the small bowel and the large bowel.

5 Intervention with a compound of the invention which reduces or prevents new endothelial cell formation into vessels (ie: neo-angiogenesis) during its earliest stages is beneficial for the treatment of inflammatory bowel disease.

10 Intervention with a compound of the invention which reduces or prevents new endothelial cell formation into vessels (ie: neo-angiogenesis) during its earliest stages could be beneficial for the prevention of the destructive arthritis that can develop in the clinical course of inflammatory bowel disease.

15 The administration of compounds of the present invention could significantly reduce the release of the pro-inflammatory chemokines (MIP-1 α , MIP-1 β and IL-8) and, in turn, the release of the pro-inflammatory cytokines (TNF- α , IL-1 and IL-6) in inflammatory bowel disease.

20 The simultaneous administration of compounds such as dendrimer gen 3.5-glucosamine and dendrimer gen 3.5-glucosamine 6-sulfate to concurrently inhibit angiogenesis and to inhibit the release of pro-inflammatory chemokines and, in turn, the release of pro-inflammatory cytokines provides for considerable synergy of action with minimal toxicity.

25 The compounds will accumulate in the inflamed tissues that are central to the pathophysiology of rheumatoid arthritis, inflammatory bowel disease and psoriasis. The endothelial cells associated with areas of inflammation and the increased vascularity caused by the development of angiogenesis will facilitate the accumulation of the compounds into the areas of inflammation.

30 Particularly advantageous features of the present invention include: the inhibition of the release of chemokines and, in turn, cytokines that results in the rejection of allogeneic organ transplants, the inhibition of the release of chemokines and, in turn, cytokines, and the angiogenesis that results in the rejection of allogeneic organ transplants, effective

maintenance of the counter inflammatory environment that is required to prevent the rejection of the transplanted organ, and effective maintenance of the counter inflammatory environment that is required to prevent the development of graft versus host disease.

5 In a preferred aspect, the present invention also provides the use of dendrimer gen. 3.5-glucosamine and/or dendrimer gen. 3.5-glucosamine sulfate in any form of allogeneic organ transplant. This includes corneal, kidney, heart, lung, liver, gut, skin and bone marrow transplantation.

10 Dendrimer gen 3.5-glucosamine 6-sulfate has a direct effect on normal endothelial cells which leads to the inhibition of new vessel formation. The administration of dendrimer gen 3.5-glucosamine 6-sulfate to patients provides a way of preventing the angiogenesis that is necessary for the establishment and the subsequent growth and development of a 15 metastatic deposit of malignant cells into a tumour especially if administered early on in the course of the disease.

20 A drug like dendrimer gen 3.5-glucosamine 6-sulfate or a combination of dendrimer gen 3.5-glucosamine and dendrimer gen 3.5-glucosamine 6-sulfate which reduces or prevents the proliferation of endothelial cells and prevents neo-angiogenesis during the earliest 25 stages of the growth of the metastatic lesion provides a way to abort or even prevent the development of the metastatic lesion.

25 A particularly preferred formulation for use in patients is dendrimer gen 3.5-glucosamine (7%) and dendrimer gen 3.5-glucosamine sulfate (7%) which is being used at 100 to 200 µg/ml.

30 Particularly preferred polymers have glucosamine or sulfated glucosamine covalently linked to the anionic dendrimer. The dendrimer gen 3.5-glucosamine and dendrimer gen 3.5-glucosamine 6-sulfate were found to downregulate the release of the chemokines macrophage inflammatory protein-1 α , macrophage inflammatory protein-1 β , and interleukin-8, and, in turn, downregulates the release of the cytokines tumour necrosis factor- α , interleukin-1 β and interleukin-6 from human peripheral blood mononuclear cells.

Compounds of the present invention such as dendrimer gen 3.5-glucosamine can also be used in combination with existing and accepted therapeutic approaches as adjuvant therapy. For example, in the sepsis syndrome they could be used with antibacterial drugs or antifungal drugs. In rheumatoid arthritis and related conditions, Behcet's disease, inflammatory bowel disease and psoriasis, they could be used with steroids and disease modifying drugs such as methotrexate. In the treatment of cancer metastases, they could be used with anti-mitotic drugs. In the treatment of allogeneic organ transplant rejection, and in graft versus host disease, they could be used with steroids and/or cyclosporin and/or FK506 and/or azathioprine.

10

The dendrimer gen 3.5-glucosamine 6-sulfate has anti-inflammatory and anti-angiogenic properties. It would be useful as adjuvant therapy in rheumatoid arthritis and related conditions, Behcet's disease, inflammatory bowel disease, psoriasis, in the treatment of cancer metastases, rejection of allogeneic organ transplants, and graft versus host disease. 15 Alternatively, the dendrimer gen 3.5-glucosamine and the dendrimer gen 3.5-glucosamine 6-sulfate could be used in combination for the treatment of each of these disorders. The simultaneous administrations of dendrimer gen 3.5-glucosamine and dendrimer gen 3.5-glucosamine 6-sulfate offers considerable synergy of action with lower doses and less frequent administration being required.

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The invention is further illustrated in the following examples.

A) CHEMISTRY AND TOXICITY :

Examples A 1) Chemical synthesis:

Starburst PAMAM anionic dendrimers generation 2.5 and 3.5 (Figure 1a & b) synthesised 25 by the divergent approach were used (Available from Dendritech Inc, Midland, MI, USA or Sigma /Aldrich, Gillingham, Dorset, England, UK). D-glucosamine hydrochloride, D-glucosamine 6-sulfate hydrochloride, D-glucosamine 3,6-disulfate sodium salt and D-glucosamine 3,4,6-trisulfate were used as the sodium salt. A known amount of the dendrimers (gen 1.5 or gen 2.5 or gen 3.5) stored as the sodium salt in methanol solution 30 were evaporated under a nitrogen stream in order to obtain 1 mmol of dendrimer sodium salt. The dendrimers were then solubilised in double deionised water and the pH reduced with hydrochloric acid (HCl) in order to transform the surface groups from the non-reactive sodium carboxylated ($\text{COO}^- \text{Na}^+$) to the reactive carboxylic acid (COOH). A known amount of glucosamine hydrochloride, glucosamine 6-sulfate, glucosamine 3,6-

disulfate, or glucosamine 3,4,6-trisulfate was solubilised in double deionised water and the pH increased with triethylamine in order to obtain the reactive free base (NH_2). In the preparation of the dendrimer-glucosamine conjugates, the reaction was spiked with a known amount of ^{14}C -glucosamine solution in double deionised water in order to follow the reaction and determine the amount of glucosamine attached to the dendrimers. The glucosamine and ^{14}C -glucosamine aqueous solution or sulfated glucosamine solutions were then added to the dendrimer solution and an acid pH maintained with HCl. Subsequently, an excess of the condensing agent 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride was solubilised in water and added to the reaction solution (Figure 2). The reaction mixture was stirred at room temperature.

Having prepared the dendrimer-glucosamine conjugates, some of the reaction mixture was passed through a PD-10 column and the fractions collected read on a β -counter to establish the presence of a high molecular weight conjugated compound. The reaction mixture was then purified in order to eliminate the unreacted glucosamine, the excess 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, and the urea formed during the reaction. In the case of dendrimers gen 3.5, the reaction mixture was dialysed against water and subsequently freeze-dried. In the case of the lower molecular weight generation dendrimers, the reaction mixture was freeze-dried, re-solubilised in deionised water and then passed through a Sephadex G15 column. The fractions at higher molecular weight containing the purified conjugate were collected, pooled together and freeze-dried.

After purification, a portion of the dendrimer-glucosamine conjugate was passed through a PD-10 column and the fractions collected read on a β -counter to monitor for the presence of unbound glucosamine. The total amount of glucosamine bound to the surface of the dendrimers was also determined using a β -counter. Characterisation and reproducibility of synthesis data for the dendrimer gen 3.5-glucosamine is shown in Table 1. In the case of the dendrimer-glucosamine sulfate conjugates, the amount of sulfated sugar attached to the polymer was determined by sulphur analysis: a known weight of the sample was combusted in an atmosphere of oxygen in a Schoniger oxygen flask. The products of combustion were washed into a 50 ml flask with double deionised water and made up to volume. This solution was analysed for sulphate content by ion chromatography using Dionex 100 ion chromatography. All of the prepared conjugates were also analysed by infrared spectroscopy. Characterisation and reproducibility of the

synthesis data for the dendrimer gen 3.5-sulfated glucosamine constructs is shown in Table 2.

All of the conjugates were treated with activated carbon to remove endotoxin (ie : LPS) prior to their biological evaluation. Endotoxin levels were determined using the limulus amebocyte lysate assay. All biological data was derived with batches of compounds whose endotoxin level was <0.1 EU/ml.

Examples A 2 : Chemical characterisation of dendrimer-glucosamine and dendrimer-sulfated glucosamine conjugates:

When glucosamine was conjugated to the dendrimers, the fractions collected by gel filtration (PD-10 column) of the reaction mixture and subsequently analysed at the β -counter showed a peak in fractions 7, 8, 9 & 10 collected (Figure 3). This peak denotes the presence of a high molecular weight structure containing ^{14}C -glucosamine confirming the formation of a dendrimer-glucosamine conjugate. FT-IR spectra peaks at 1640 cm^{-1} and 1560 cm^{-1} , in conjugation with the peak at 3260 cm^{-1} indicate the formation of the amide bond (Figure 4 and Figure 5). In the case of dendrimer-sulfated glucosamine conjugates, the further peak at 1210 cm^{-1} (Figure 6 for the dendrimer-glucosamine 6-sulfate conjugate) indicates the presence of sulfated glucosamine with prevention of loss of sulfuric acid during the conjugation.

The amount of glucosamine linked to the dendrimers, expressed as a percentage of converted carboxylic groups, ranged from 1.5 to 10.1 %. In the case of the sulfated dendrimers, the sulphur content increased in the following order: monosulfated sugar (2.79 % m:m S); disulfated sugar (5.78 % m:m S); trisulfated sugar (7.93 % m:m S). From the sulphur content, it was possible to calculate the amount of sulfate present in the conjugate as being 6 % in the case of glucosamine 6-sulfate, 11 % in the case of glucosamine 3,6-disulfate and 15 % in the case of the glucosamine 3,4,6-trisulfate conjugate.

The one-step reaction was also investigated as a feasible method for the conjugation of:-
a) PAMAM anionic dendrimers gen 2.5 to molecules containing aminic groups as well as to glucosamine 6-sulfate. The presence of characteristic peaks for the amide bond (3270

cm⁻¹, 1640 and 1550 cm⁻¹) indicated the formation of a conjugate between dendrimers and glucosamine or sulfated glucosamine as was seen with the generation 3.5 dendrimers.

5 Molecules described above, can be attached to the anionic PAMAM dendrimers using the same chemistry. This has been successfully achieved for 3-amino-1-propanesulfonic acid as an example.

10 It is known that guest molecules can become trapped in the core of the dendrimer (Kempen HJM, Hoes C, van Boom JM, Spanjer HH, de Lange J, Langendoen A & van Berkel TJC. (1984) A water-soluble cholestryl-containing trisgalactoside - synthesis, properties, and use in directing lipid-containing particles to the liver. Journal Medicinal Chemistry 27, 1306-1312). Therefore it was important to determine the amount of unbound glucosamine inside the dendritic structure. This was undertaken by keeping a known amount of the conjugate in solution over a period of time and then determining the 15 amount of unbound glucosamine present by gel filtration. Figure 7 (i) shows the gel filtration profiles of the conjugate stored in aqueous solution compared to free glucosamine; the amount of unbound glucosamine increased only slightly over time from 3.1 wt % at day 0 to 7.5 wt % after 90 days of storage at 4°C. Therefore, it was concluded that the covalent amide bond is stable and that there was no significant 20 entrapment of free glucosamine within the dendrimer. Similar results were obtained from the dendrimer-glucosamine gen 2.5 after 30 days of storage under similar conditions (Figure 7(ii)).

25 These observations allow a calculation of the number of molecules of glucosamine or sulfated glucosamine that have been linked to 1 molecule of dendrimer because all of the glucosamine or glucosamine sulfate molecules are linked to the outer surface of the dendrimer. The number of molecules of sulfated sugar linked to 1 molecule of dendrimer did not change with increasing numbers of sulfated groups attached to the glucosamine moiety, showing that the one-step reaction can be used as a standard method for 30 conjugating sulfated glucosamine derivatives to dendrimers. The number of molecules of glucosamine or sulfated glucosamine linked to 1 molecule of dendrimer generation 2.5 is lower when compared to generation 3.5. Therefore, due to the higher loading and the minimal toxicity of the generation 3.5 dendrimers, these conjugates were chosen as candidates for further biological investigation.

Examples A 3: Toxicity of compounds in human cell lines:

The compounds synthesised were evaluated for their toxicity in the following cell lines:-
the human T-cell line (Sup-T1), a human T-cell line transformed with HTLV-1 (C8166),
the human monocytic cell line (U937), the brain cell line transfected with human CD4
5 and the human chemokine receptor CCR5 (U87-CD4-CCR5), and the murine melanoma
cell line (B16F10).

Dendrimers, glucosamine, glucosamine 6-sulfate and dendrimer conjugates were
incubated with each cell line. Sup-T1, C8166, U937, U87-CD4-CCR5 and B16F10 cells
10 were seeded at a density of 2×10^5 cell/ml and B16F10 cells were seeded at 1×10^5
cell/ml in 96 well microtiter plates in medium containing RPMI 1640, 20 mM L-
glutamine, penicillin [250 IU/ml], streptomycin [250 µg/ml] and 10 % fetal calf serum.
The conjugates or controls were incubated for 67 h with the cells at concentrations
15 ranging from 0 – 5,000 µg/ml. An MTT assay was then used to determine cell viability
and the results were expressed as a percentage of that for cells grown in the absence of
any compound.

Dextran and poly(L-lysine) were used as negative and positive controls respectively.
Glucosamine and glucosamine 6-sulfate showed no toxicity at concentrations up to 1,000
20 µg/ml. Higher concentrations of these compounds could not be evaluated because
glucosamine interferes with the MTT assay above his concentration. Dendrimers gen 3.5
and dendrimers gen 2.5 showed no toxicity up to concentrations of 5,000 µg/ml. The
dendrimer gen 3.5-glucosamine conjugate showed IC₅₀ values ranging from 1,825 to
3,000 µg/ml in the different cell lines. The dendrimer gen 3.5-glucosamine 6-sulfate
25 conjugate had IC₅₀ values ranging from 246 – 307 µg/ml (Table 3).

Examples A 4: Toxicity of compounds against primary human cells in vitro:

The dendrimers and the dendrimer conjugates were incubated with freshly isolated,
human peripheral blood mononuclear (PBMN) cells and with freshly isolated human
30 monocyte-derived-macrophages (MDM).

Human PBMN cells and human MDMs were separated from buffy coat residues within
24 h of collection by density gradient centrifugation. The cells were then washed with

phosphate-buffered saline (PBS), re-suspended in lymphocyte growth medium (RPMI 1640, 20 mM L-glutamine, penicillin [250 IU/ml], streptomycin [250 µg/ml] and 15 % foetal calf serum) containing human recombinant interleukin 2 (IL-2, 20 IU/ml) for 3 days and then plated in a 96 well plate at a density of 1×10^6 cells/ml. To separate 5 MDMs, the remaining cells were re-suspended in macrophage-growth medium (RPMI 1640, 20 mM L-glutamine, penicillin [250 IU/ml], streptomycin [250 µg/ml] and 10 % human serum) and plated in a plastic dish for 2 h. MDMs were then scraped, washed and plated in a 96 well plate at a density of 1×10^6 cells/ml. The monocytes were allowed to differentiate to MDMs by a further 3 days of adherence. The compounds were then added 10 to PBMN cells or to MDMs (1×10^6 cells/ml) and incubated for 67 h. Cell viability was assessed using the MTT assay at 72 h.

Dendrimer gen 3.5 and glucosamine 6-sulfate were not toxic to PBMN cells or to MDMs up to the maximum concentration of 1,000 µg/ml that was evaluated. The IC₅₀ value for 15 dendrimer gen 3.5-glucosamine was 340-360 µg/ml in primary cells as determined using an MTT assay. The IC₅₀ value for the dendrimer gen 3.5-glucosamine 6-sulfate construct, the dendrimer gen 3.5-glucosamine 3,6-disulfate construct, and the dendrimer gen 3.5-glucosamine 3,4,6-trisulfate conjugate was 230-260 µg/ml in primary cells as determined using an MTT assay.

20 Examples A 5: The effect of the dendrimer gen 3.5-glucosamine and the dendrimer gen 3.5 sulfated glucosamine conjugates on the release of MIP-1 β from single donor, human PBMN cells, MDMs and peritoneal macrophages:

Recent studies have shown that macrophage inflammatory protein-1 β (MIP-1 β) is the 25 earliest pro-inflammatory chemokine that is released by cells of macrophage origin after they have been exposed to an inflammatory stimulus (Wang Z-M, Liu C & Dziarski R. Chemokines are the main pro-inflammatory mediators in human monocytes activated by *Staphylococcus aureus*, peptidoglycan, and endotoxin. (2000) J. Biological Chemistry 275; 20260-20267). All the polymers that have been studied to date, irrespective of 30 whether they have been derived from a natural source or have been synthetically made have shown a significant ability to induce the release of pro-inflammatory cytokines and chemokines from immune cells. We therefore exposed single donor human PBMN cells, single donor human MDMs, and single donor human peritoneal macrophages to the

compounds synthesised in order to determine whether they induced any pro-inflammatory responses from these cells.

Cells were isolated as previously described within 4 hours of collection and then plated at 5 1×10^6 cells/ml. Dendrimer gen 3.5, dendrimer gen 3.5 glucosamine, dendrimer gen 3.5-glucosamine 6-sulfate, dendrimer gen 3.5-glucosamine 3,6-disulfate, and dendrimer gen 3.5-glucosamine 3,4,6-trisulfate were then added to the cells at a concentration of 50 to 200 $\mu\text{g}/\text{ml}$. Cell free culture supernatants were harvested 36 h later and assayed for MIP-200 $\mu\text{g}/\text{ml}$. Cell free culture supernatants were harvested 36 h later and assayed for MIP-1 β .

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No release of MIP-1 β above that seen in the control wells occurred with:

- a) PBMN cells and dendrimer gen 3.5, dendrimer gen 3.5 glucosamine, or dendrimer gen 3.5-glucosamine 6-sulfate at 50 $\mu\text{g}/\text{ml}$ (Figure 8(i)).
- b) MDMs and dendrimer gen 3.5, dendrimer gen 3.5 glucosamine, or dendrimer gen 3.5-glucosamine 6-sulfate at 50 $\mu\text{g}/\text{ml}$ (Figure 8(ii) and Figure 8(iii)).
- c) MDMs and dendrimer gen 3.5-glucosamine 3,6-disulfate, and dendrimer gen 3.5-glucosamine 3,4,6-trisulfate at 50 $\mu\text{g}/\text{ml}$ (Figure 9).
- d) Peritoneal macrophages after 36 h (Figure 10(i) and Figure 10(ii)) and after 72 h (Figure 10(iii)) of culture with dendrimer gen 3.5, dendrimer gen 3.5 glucosamine, or dendrimer gen 3.5-glucosamine 6-sulfate with each molecule at 25 $\mu\text{g}/\text{ml}$.
- e) Peritoneal macrophages after 36 h of culture with dendrimer gen 3.5, dendrimer gen 3.5 glucosamine or dendrimer gen 3.5-glucosamine 6-sulfate with each molecule at 50 $\mu\text{g}/\text{ml}$ (Figure 11(i) and Figure 11(ii) respectively).
- f) Peritoneal macrophages after 36 h of culture with dendrimer gen 3.5-glucosamine 3,6-disulfate or dendrimer gen 3.5-glucosamine 3,4,6-trisulfate with each molecule at 50 $\mu\text{g}/\text{ml}$ (Figure 12(i) and Figure 12(ii) respectively).
- g) Peritoneal macrophages after 36 h of culture with dendrimer gen 3.5-glucosamine 3,6-disulfate or dendrimer gen 3.5-glucosamine 3,4,6-trisulfate with each molecule at 100 $\mu\text{g}/\text{ml}$ (Figure 13(i) and Figure 13(ii)) and at 200 $\mu\text{g}/\text{ml}$ (Figure 13(iii) and Figure 13(iv)).

Therefore, in conclusion, it is important to note that dendrimer gen 3.5-glucosamine, dendrimer gen 3.5-glucosamine 6-sulfate, dendrimer gen 3.5-glucosamine 3,6-disulfate,

and dendrimer gen 3.5-glucosamine 3,4,6-trisulfate did not affect the release of chemokines or cytokines from immune cells (ie: primary human peripheral blood mononuclear cells, human monocyte derived macrophages or human peritoneal macrophages) that were in their normal resting state. This is the first time that a 5 glycodendrimer has been shown not to affect human primary cells in their normal resting state. Therefore when administered as a medicine, these molecules will only have a drug mediated effect on diseased human cells. These two compounds will not interfere with the routine homeostatic control under which normal human cells operate on a day-to-day basis.

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EXAMPLES B : EXPERIMENTS WITH LIPOPOLYSACCHARIDE (LPS) :

The effect of the dendrimer gen 3.5-glucosamine conjugate on the release of chemokines and, in turn, cytokines by LPS from single donor human PBMN cells:

PBMN cells were isolated from fresh blood from single donors by density gradient 15 centrifugation. The cells were re-suspended in growth medium (RPMI 1640, 20 mM L-glutamine, penicillin [250 IU/ml], streptomycin [250 µg/ml] and 10 % endotoxin free human serum) and the cell density adjusted to 2×10^6 cells/ml. Aliquots of the cell suspension were transferred to a 12-well tissue culture plate and incubated for 15 min at 20 37°C. To these PBMN cells, LPS was added (usually at 5 ng/ml) either before or after the addition of the endotoxin free dendrimer gen 3.5-glucosamine. In the first set of experiments, the LPS (Salmonella minnesota, Sigma. Catalogue number L9764) was added 30 min to 24 h after the dendrimer gen 3.5-glucosamine conjugate. In the second 25 set of experiments, the LPS was added 30 min, 1 h, 2 h, 3 h or 4 h before the dendrimer gen 3.5-glucosamine. The concentration of dendrimer gen 3.5-glucosamine added was 150 – 200 µg/ml. The cells were then maintained at 37°C with 5% CO₂. Cell free supernatants were collected at regular time points up to a maximum of 96 h later and assayed for macrophage inflammatory protein-1α (MIP-1α), macrophage inflammatory protein-1β (MIP-1β), Interleukin-8 (IL-8), Tumor Necrosis Factor-α (TNF-α), Interleukin-1β (IL-1β) and Interleukin-6 (IL-6).

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Experiment B 1: Single donor MDMs were prepared as described and cultured with dendrimer gen 3.5-glucosamine up to a concentration of 150 µg/ml for 72 h. No reduction

in cell viability was seen when compared to MDMs cultured in media alone over a period of 72 h (Figure 14(i)).

Single donor, human umbilical vein endothelial cells (HUVECs) were also prepared as follows. Umbilical cords were obtained from placentas from the maternity wards of Hammersmith and Queen Charlotte's Hospitals within 4 h of delivery. Excess blood was removed from the cord by massaging the outside. The cord was then sprayed with 70% alcohol and wiped. The ends were cut with sharp scissors and the umbilical vein identified. 14 gauge cannulas with luer lock ends were inserted into each end of the umbilical vein and tied in place. The vein was then flushed through with HBSS (Sigma, Poole, Dorset) using a 20 ml syringe attached to a cannula. Endothelial cells were dislodged from the vein using a solution of 20 mg/ml collagenase A (Sigma) in HBSS (20 ml) and filter sterilised using a 20 μ m membrane. Using a second 20 ml syringe, this solution was introduced into the umbilical vein and carefully agitated by gently applying pressure to each syringe in turn. The cord was then incubated in a water bath at 37°C for 20 min with a second agitation for 10 min. The collagenase A solution was removed from the cord and added to 20 ml of media containing 20% FCS to quench the enzyme and a further 20 ml of media flushed through the vein to remove any remaining endothelial cells. Cells were pelleted by centrifugation (400g/5 min), resuspended in 5 ml growth media [MEM alpha (Sigma)] containing 20% FCS, L-glutamine (2 mM), 200 IU/ml penicillin, 200 μ g/ml streptomycin and 50 μ g/ml clinical grade heparin (Leo Laboratories, Bucks). They were then transferred to collagen coated 25 cm² tissue culture flasks and incubated at 37°C with 5% CO₂. After 48 h incubation, 5 μ g/ml endothelial cell growth supplement (ECGS - Sigma) was added. Cultures were examined daily using an inverted microscope. When confluent, growth medium was aspirated and replaced with an equal volume of HBSS at 37°C. Cells were then removed by trypsinisation. The HBSS was aspirated and replaced with 2 ml trypsin (0.5 mg/ml)/EDTA (0.2 mg/ml) solution in HBSS (Life Technologies, Paisley, UK) and incubated at 37°C for 5-10 min until all cells had been removed from the bottom of the flask. Cells were then split to a ratio of 1:2 to 1:3 with fresh growth media containing ECGS (5 μ g/ml) and transferred to the appropriate flask or tissue culture plate depending upon the assay being performed.

The HUVECs were cultured with dendrimer gen 3.5-glucosamine up to a concentration of 100 μ g/ml for 72 h. No reduction in cell viability was seen when compared to HUVECs cultured in media alone over a period of 72 h (Figure 14(ii)).

5 Experiment B 2: Single donor PBMN cells were isolated and resuspended in RPMI, L-glutamine, penicillin, streptomycin and 10% human serum at a density of 1×10^6 cells/ml. The cells were then plated (1 ml) in 12 well tissue culture plates and then cultured for 15 min at 37°C in 5% CO₂. LPS was then added at 5 ng/ml. Cell free culture supernatants were harvested at regular intervals for up to 21 h and assayed for MIP-1 β as shown in Figure
10 15.

15 Experiment B 3: Single donor PBMN cells were isolated from one individual, resuspended in RPMI, L-glutamine, penicillin, streptomycin and 10% human serum at a density of 1×10^6 cells/ml. The cells were then plated (1 ml) in 12 well tissue culture plates and cultured for 15 min at 37°C in 5% CO₂. Dendrimer gen 3.5-glucosamine was then added at 100 μ g/ml. The cells were cultured for 30 min at 37°C in 5% CO₂ and LPS was then added at 5 ng/ml. Cell free culture supernatants were harvested 21 h later and assayed for MIP-1 β as shown in Figure 16(i). A significant reduction in MIP-1 β release was seen when dendrimer gen 3.5-glucosamine was present.

20 It has been confirmed that dendrimer gen 3.5-glucosamine and dendrimer gen 3.5-glucosamine 6-sulfate are not mediating their activity by binding to the endotoxin used in these experiments.

25 Experiment B 4: Single donor PBMN cells were isolated and resuspended in RPMI, L-glutamine, penicillin, streptomycin and 10% human serum at a density of 1×10^6 cells/ml. The cells were then plated (1 ml) in 12 well tissue culture plates and cultured for 15 min at 37°C in 5% CO₂. Dendrimer gen 3.5-glucosamine was then added at 100 μ g/ml. The cells were cultured for 1 hour at 37°C in 5% CO₂ and LPS was then added at 5 ng/ml. Cell free culture supernatants were harvested 21 h later and assayed for MIP-1 β as shown in Figure
30 16(ii). A significant reduction in MIP-1 β release was seen when dendrimer gen 3.5-glucosamine was present.

Experiment B 5: Single donor PBMN cells were isolated and resuspended in RPMI, L-glutamine, penicillin, streptomycin and 10% human serum at a density of 1×10^6 cells/ml. The cells were then plated (1 ml) in 12 well tissue culture plates and cultured for 15 min at 37°C in 5% CO₂. Dendrimer gen 3.5-glucosamine was then added at 100 µg/ml. The cells 5 were cultured for 24 hours at 37°C in 5% CO₂ and LPS was then added at 5 ng/ml. Cell free culture supernatants were harvested 21 h later and assayed for MIP-1β as shown in Figure 16(iii). A significant reduction in MIP-1β release was seen when dendrimer gen 3.5-glucosamine was present.

10 Experiment B 6: Single donor PBMN cells were isolated and resuspended in RPMI, L-glutamine, penicillin, streptomycin and 10% human serum at a density of 1×10^6 cells/ml. The cells were then plated (1 ml) in 12 well tissue culture plates and cultured for 15 min at 37°C in 5% CO₂. Dendrimer gen 3.5-glucosamine was then added at 100 µg/ml. The cells 15 were cultured for periods ranging from 30 minutes to 24 hours at 37°C in 5% CO₂ and LPS was then added at 5 ng/ml. Cell free culture supernatants were harvested 21 h later and assayed for MIP-1β as shown in Figure 16(iv). A significant reduction in MIP-1β release was seen when dendrimer gen 3.5-glucosamine was present at all time points studied.

20 Experiment B 7: Single donor PBMN cells were isolated and resuspended in RPMI, L-glutamine, penicillin, streptomycin and 10% human serum at a density of 1×10^6 cells/ml. The cells were plated (1 ml) in 12 well tissue culture plates and then cultured for 15 min at 37°C in 5% CO₂. LPS was then added at 5 ng/ml. Cell free culture supernatants were harvested at regular intervals for up to 21 h and assayed for TNF-α as shown in Figure 17.

25 Experiment B 8: Single donor PBMN cells were isolated and resuspended in RPMI, L-glutamine, penicillin, streptomycin and 10% human serum at a density of 1×10^6 cells/ml. The cells were then plated (1 ml) in 12 well tissue culture plates and cultured for 15 min at 37°C in 5% CO₂. Dendrimer gen 3.5-glucosamine was then added at 100 µg/ml. The cells 30 were cultured for 30 min at 37°C in 5% CO₂ and LPS was then added at 5 ng/ml. Cell free culture supernatants were harvested 21 h later and assayed for MIP-1α as shown in Figure 18(i), MIP-1β as shown in Figure 18(ii), IL-8 as shown in Figure 18(iii), TNF-α as shown in Figure 18(iv), IL-1β as shown in Figure 18(v), and IL-6 as shown in Figure 18(vi). For

all of the chemokines and pro-inflammatory cytokines studied, a significant reduction in their release was seen when dendrimer gen 3.5-glucosamine was present.

5 Experiment B 9: Single donor PBMN cells were isolated and resuspended in RPMI, L-glutamine, penicillin, streptomycin and 10% human serum at a density of 1×10^6 cells/ml. The cells were then plated (1 ml) in 12 well tissue culture plates and cultured for 15 min at 37°C in 5% CO₂. Dendrimer gen 3.5-glucosamine was then added at 200 µg/ml. The cells were cultured for 30 min at 37°C in 5% CO₂ and LPS was then added at 5 ng/ml. Cell free culture supernatants were harvested 21 h later and assayed for MIP-1 α as shown in Figure 19(i), MIP-1 β as shown in Figure 19(ii), IL-8 as shown in Figure 19(iii), TNF- α as shown in Figure 19(iv), IL-1 β as shown in Figure 19(v), and IL-6 as shown in Figure 19(vi). For 10 all of the chemokines and pro-inflammatory cytokines studied, a significant reduction in their release was seen when dendrimer gen 3.5-glucosamine was present.

15 Experiment B 10: Single donor PBMN cells were isolated and resuspended in RPMI, L-glutamine, penicillin, streptomycin and 10% human serum at a density of 1×10^6 cells/ml. The cells were then plated (1 ml) in 12 well tissue culture plates and cultured for 15 min at 37°C in 5% CO₂. Dendrimer gen 3.5-glucosamine was then added at 200 µg/ml. The cells were cultured for 1 hour at 37°C in 5% CO₂ and LPS was then added at 5 ng/ml. Cell free culture supernatants were harvested 21 h later and assayed for MIP-1 β as shown in Figure 20(i) and TNF- α as shown in Figure 20(ii). For 20 all of the chemokines and pro-inflammatory cytokines studied, a significant reduction in their release was seen when dendrimer gen 3.5-glucosamine was present.

25 Experiment B 11: Single donor PBMN cells were isolated and resuspended in RPMI, L-glutamine, penicillin, streptomycin and 10% human serum at a density of 1×10^6 cells/ml. The cells were then plated (1 ml) in 12 well tissue culture plates and cultured for 15 min at 37°C in 5% CO₂. LPS was then added at 5 ng/ml and the cells were cultured for 30 min, or 1 hour, or 2 hours, or 3 hours, or 4 hours at 37°C in 5% CO₂ before adding dendrimer gen 30 3.5-glucosamine at 100 µg/ml. Cell free culture supernatants were harvested 21 h after the addition of the LPS and assayed for MIP-1 α as shown in Figure 21(i), MIP-1 β as shown in Figure 21(ii), IL-8 as shown in Figure 21(iii), TNF- α as shown in Figure 21(iv), IL-1 β as shown in Figure 21(v), and IL-6 as shown in Figure 21(vi). For all of the chemokines

and pro-inflammatory cytokines studied, a significant reduction in their release was seen when dendrimer gen 3.5-glucosamine was present.

Experiment B 12: Single donor PBMN cells were isolated and resuspended in RPMI, L-5 glutamine, penicillin, streptomycin and 10% human serum at a density of 1×10^6 cells/ml. The cells were then plated (1 ml) in 12 well tissue culture plates and cultured for 15 min at 37°C in 5% CO₂. Dendrimer gen 3.5-glucosamine in which the loading of glucosamine on the generation 3.5 dendrimer was 3 % (rather than the 7 % loading used in all the other experiments described) was then added at a concentration of 150 µg/ml or 300 µg/ml. The 10 cells were cultured for 30 min at 37°C in 5% CO₂ and LPS was then added at 5 ng/ml. Cell free culture supernatants were harvested 22 h later and assayed for MIP-1 β as shown in Figure 22(i), or for TNF- α as shown in Figure 22(ii). For all of the chemokines and pro-inflammatory cytokines studied, a significant reduction in their release was seen when dendrimer gen 3.5-glucosamine was present.

15 **EXAMPLES C - EXPERIMENTS WITH THE MIXING OF HUMAN CELLS**
FROM DIFFERENT DONORS:

The effect of the dendrimer gen 3.5-glucosamine conjugate on the release of chemokines and, in turn, cytokines after the mixing of human PBMN cells from several donors:

20 Experiment C 13: Single donor PBMN cells were isolated from 3 individuals and the cell density adjusted to 1×10^6 cells/ml. The cells from all 3 donors were then mixed together for 72 h in a 12-well tissue culture plate. The dendrimer gen 3.5-glucosamine was then added at 50 µg/ml. Cell free culture supernatants were harvested 1 day, 3 days and 4 days later and assayed for MIP-1 β and TNF- α . A significant reduction in MIP-1 β and TNF- α 25 release into the culture supernatant was seen at all 3 time points assayed as shown in Figure 23(i) and 23 (ii) respectively.

30 Experiment C 14: Single donor PBMN cells were isolated from 2 individuals and the cell density adjusted to 1×10^6 cells/ml. The cells were cultured separately for 24 h and then mixed together for 72 h in a 12-well tissue culture plate. The dendrimer gen 3.5-glucosamine was then added at 50 µg/ml. Cell free culture supernatants were harvested 1 day, 3 days and 4 days later and assayed for MIP-1 β . A significant reduction in MIP-1 β

release into the culture supernatant was seen at all 3 time points assayed as shown in Figure 23(iii).

5 Experiment C 15: Single donor PBMN cells were isolated from 2 individuals and the cell density adjusted to 1×10^6 cells/ml. The cells were mixed together and dendrimer gen 3.5-glucosamine added at 100 $\mu\text{g}/\text{ml}$. LPS was added immediately afterwards at 10 ng/ml and cell free culture supernatants harvested at regular intervals for up to 48 h. A significant reduction in MIP-1 β release into the culture supernatant was seen from 24 h onwards as shown in Figure 24(i).

10 Experiment C 16: Single donor PBMN cells were isolated from 2 individuals and the cell density adjusted to 1×10^6 cells/ml. The cells were mixed together and dendrimer gen 3.5-glucosamine added at 100 $\mu\text{g}/\text{ml}$. LPS was added 24 hours later at a concentration of 5 ng/ml. Cell free culture supernatants were harvested at regular intervals for up to 96 h. A 15 significant reduction in MIP-1 β release into the culture supernatant was seen from 24 h onwards as shown in Figure 24(ii).

EXAMPLES D : CYTOKINE AND CHEMOKINE ASSAYS :

20 Quantitation of MIP-1 β and other chemokines and cytokines in tissue culture supernatants:

Levels of MIP-1 β were measured in cell free culture supernatants using a commercially available enzyme immuno-assay (EIA; Quantikine, R&D Systems). Dendrimers gen 3.5-glucosamine conjugates were first confirmed not to interfere with the assay by mixing rhMIP-1 β at a final concentration of 125 pg/ml with the dendrimer gen 3.5-glucosamine conjugate at a final concentration of 200 $\mu\text{g}/\text{ml}$ in growth medium. The solutions were incubated for 4 h 25 at 37°C and then run as samples in the EIA. There was no interference of the dendrimer gen 3.5-glucosamine with the MIP-1 β assay (Table 4).

30 There was no interference of the dendrimer gen 3.5-glucosamine with the MIP-1 α , IL-8, IL-1 β and IL-6 assays (Table 2). In the case of TNF- α a low level interference was seen and this has been corrected for in the results shown in the figures (Table 2).

EXAMPLES E : OTHER BIOLOGICAL DATA :

Examples E1 : Preliminary animal toxicology:

Anionic dendrimers gen. 3.5 are not toxic to mice at 90 mg/kg. Four Wistar rats were injected intravenously with dendrimer gen 3.5-glucosamine at a concentration of 30 mg/kg. This is equivalent to 6 mg/200g rat. All animals were healthy at 24 h. They were 5 then killed and examined. No gross pathology was seen in any of the organs.

Examples E 2: Anticoagulant activity:

The anticoagulant activity of each compound (ie: dendrimer gen 3.5, dendrimer gen 3.5 glucosamine, dendrimer gen 3.5-glucosamine 6-sulfate, dendrimer gen 3.5-glucosamine 10 3,6-disulfate, and dendrimer gen 3.5-glucosamine 3,4,6-trisulfate) was determined using the following in vitro assays:- kaolin partial thromboplastin time, prothrombin time, thrombin time and a Factor Xa assay. No anticoagulant activity was seen at the maximum concentration of 200 µg/ml that was analysed for all of the compounds tested. For dendrimer gen 3.5 glucosamine, this was also confirmed at a concentration of 300 µg/ml. 15 The results are shown in Table 5. This is the first time that a sulphated polysaccharide has been made which has biological activity but no anticoagulant activity.

Examples E 3: Anti-HIV-1 activity of dendrimer-sulfated glucosamine constructs:

The anti-HIV-1 activity of dendrimers gen 3.5 glucosamine and the sulfated glucosamine 20 conjugates synthesised was determined using C8166 cells and the HIV-1.IIIb virus up to a concentration of 100 µg/ml. No anti-HIV-1 activity was seen.

EXAMPLES F: BIOLOGICAL ACTIVITY OF DENDRIMER SULPHATED GLUCOSOAMINE:

25 Examples F 1: Dendrimer gen 3.5-glucosamine 6-sulfate does not affect the growth characteristics of human PBMN cells, human MDMs or HUVECs at the concentrations used:

The dendrimer gen 3.5-glucosamine 6-sulfate did not affect cell viability or the growth characteristics of PBMN cells (Figure 25(i) and Figure 25(ii)), or of MDMs (Figure 26) 30 when present at a concentration of up to 150 µg/ml in cultures maintained for up to 5 days. Cell viability was determined by Trypan blue exclusion and an MTT assay. Furthermore, the dendrimer gen 3.5-glucosamine 6-sulfate construct was not toxic to

HUVECs when added to cultures of these cells up to a concentration of 100 µg/ml for up to 72 hours (Figure 27(i) and Figure 27(ii) and Figure 27(iii)).

Examples F 2: The effect of dendrimer gen 3.5-glucosamine 6-sulfate on the release of chemokines and cytokines by LPS from single donor human PBMN cells:

- 5 Single donor PBMN cells were isolated, resuspended in RPMI, L-glutamine, penicillin, streptomycin and 10% human serum at a density of 1×10^6 cells/ml. The cells were then plated (1 ml) in 12 well tissue culture plates and cultured for 15 min at 37°C in 5% CO₂. Dendrimer gen 3.5-glucosamine 6-sulfate was then added at 150 µg/ml or at 200 µg/ml.
- 10 The cells were cultured for 30 min at 37°C in 5% CO₂ and LPS (5 ng/ml) added. Cell free culture supernatants were harvested 24 h later and assayed for MIP-1β as shown in Figure 28(i). A significant reduction in MIP-1β release was seen when dendrimer gen 3.5-glucosamine 6-sulfate was present.
- 15 Cell free culture supernatants were also harvested and assayed for TNF-α as shown in Figure 28(ii). A significant reduction in TNF-α release was seen when dendrimer gen 3.5-glucosamine 6-sulfate was present at 150 µg/ml or at 200 µg/ml.

- Examples F 3: The effect of dendrimer gen 3.5-glucosamine 6-sulfate on the release of chemokines and cytokines after the mixing of human MDMs from four donors:
- 20 Single donor PBMC cells were isolated from 4 individuals by density gradient centrifugation and then mixed together for 24 h. The cells were re-suspended in growth medium (RPMI 1640, 20 mM L-glutamine, penicillin [250 IU/ml], streptomycin [250 µg/ml] and 10 % human serum) and the cell density adjusted to 2×10^6 cells/ml. To separate MDMs, the cells were plated in a plastic dish for 2 h and the MDMs then scraped, washed, and resuspended in growth medium. They were then re-plated at a density of 1×10^6 cells/ml and left in culture for 72 hours before adding either dendrimer gen 3.5-glucosamine or dendrimer gen 3.5-glucosamine 6-sulfate at a concentration of 25 µg/ml. Cell free culture supernatants were harvested 36 h later and assayed for MIP-1β as shown in Figure 29. A significant reduction in MIP-1β release was seen when dendrimer gen 3.5-glucosamine was present as well as when dendrimer gen 3.5-glucosamine 6-sulfate was present.

Examples F 4: Endothelial microtubule formation assay to determine the anti-angiogenic activity of compounds:

5 Kubota et al. reported that human umbilical vein endothelial cells undergo morphological differentiation with tube formation when cultured on a reconstituted gel composed of basement membrane proteins (Matrigel; Beckton-Dickinson) (Kubota, Y., H.K. Kleinman, G.R. Martin, and T.J. Lawley. 1988. Role of laminin and basement membrane in the morphological differentiation of human endothelial cells into capillary-like structures. *J. Cell. Biol.* 107: 1589-1598). This has parallels with the *in vivo* situation where endothelial cells line the lumen of blood vessels and are in contact with the 10 extracellular matrix of basement membranes that are composed of collagen IV, heparan sulphate proteoglycan and the glycoproteins laminin and nidogen/entactin.

15 Due to the ability of basement membranes to stimulate differentiation, cells plated onto the gel attach rapidly. Within one to two hours, elongated processes are observed. After eight hours, the endothelial cell cultures show abundant networks of branching and anastomosing of cords of cells. By eighteen hours, the endothelial cells have formed an interconnected network of anastomosing cells which, by low power light microscopy, have a honeycomb appearance. The tube like structures formed by endothelial cells on 20 Matrigel persist for several days. The formation of the tube structures is not dependent on extracellular growth factors or the presence of heparin in the culture media. Tube formation also seems to be relatively specific for endothelial cells because neither human dermal fibroblasts nor human smooth muscle cells form tubes when cultured on Matrigel.

25 Ultrastructural EM studies have confirmed that the anastomosing cytoplasmic extensions of the morphologically differentiated endothelial cells contain a lumen that is completely encircled by one or two endothelial cells in cross section. The lumen contains various amounts of degenerated cytoplasm, suggesting that very rapid remodelling of the cell takes place during tube formation. Viability studies of endothelial cells cultured on Matrigel do not indicate that cell death plays an important role in tube formation. 30 Moreover, these differentiated cells retain the characteristic Weibel-Palade bodies of endothelial cells.

The endothelial microtubule formation assay was performed by isolating human umbilical vein endothelial cells from umbilical cords within 6 h of delivery by caesarean section.

The umbilical vein was cannulated and 0.1% collagenase in phosphate buffered saline was introduced and incubated for 20 minutes. The endothelial cells liberated by the collagenase were obtained by washing the umbilical vein with Medium 199. The cells were washed three times with Medium 199 and then cultured in tissue culture flasks 5 coated with fibronectin. Growth media consisted of Medium 199 with 20% foetal bovine serum, 30 µg/ml endothelial cell growth supplement, 10 IU/ml heparin, 100 IU/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. Human umbilical vein endothelial cells were passaged at confluence after treatment with trypsin-EDTA. All cells were used at passages 3 to 6.

10 Aliquots of Matrigel were dispensed into 35 mm diameter tissue culture dishes on ice and then incubated at 37°C for 10 min to allow the gel to set. Dendrimer gen 3.5-glucosamine 6-sulfate was prepared in HUVEC growth medium and added to the dishes at several 15 different concentrations. HUVEC's at passage 3 to 6 were harvested using trypsin and suspended in growth medium at a density of 2 - 3 x10⁵ cells/ml. One ml aliquots of the cell suspension were added to the plates containing the dendrimer gen 3.5-glucosamine 6-sulfate and incubated at 37°C, 5% CO₂. At various time points from 8 - 24 hours, the plates were examined and scored using a published scheme from 0 - 4, where 0 = all 20 adherent cells remain single, and 4 = all adherent cells involved in tubular structures. New vessel formation was quantified blind by three different observers at 18 h. The within-observer and between-observer variability was <10% (n = 4). The results are shown in Table 6 and photographs of the assay in Figures 30 to 32. Dendrimer gen 3.5-glucosamine 6-sulfate significantly reduced the level of tube formation at 12 µg/ml and abolished it completely at 75 µg/ml to 100 µg/ml.

25 The in vitro model of migration and microvessel formation used for testing dendrimer gen 3.5-glucosamine 6-sulfate fulfilled the two hallmarks of angiogenesis; ie: endothelial cell proliferation and capillary sprouting. Our in vitro observations show that dendrimer gen 3.5-glucosamine 6-sulfate inhibits new vessel formation by normal endothelial cells and 30 that this prevents new blood vessel formation; ie: angiogenesis.

Examples F 5: In vitro angiogenesis assay:

Blood vessels (approximately 1-2 mm diameter) were excised from the apical surface of human placentas within 6 h of an elective Caesarian birth. The use of the placentas was

approved by the Ethics Committee of Hammersmith Hospitals Trust, London. The blood vessels were placed in Hank's balanced salt solution and cut into 1-2 mm fragments using fine dissecting forceps and iridectomy scissors. Residual clots were removed and the blood vessels then soaked in Hank's balanced salt solution.

5 The effect of each compound on new blood vessel formation was determined by culturing the blood vessels within a fibrin clot in 24-well tissue culture plates. Thirty μ l of bovine thrombin (50 NIH U/ml in 0.15 M sodium chloride) was added to each well followed by 10 1 ml/well of 3 mg/ml bovine fibrinogen in Medium 199. The thrombin and fibrinogen were rapidly mixed and a blood vessel fragment placed in the centre of the well. Fibrin gel formation occurred rapidly and left the vessel suspended within the gel. One ml/well of Medium 199 supplemented with 20% foetal calf serum, 250 IU/ml penicillin and 250 15 U/ml streptomycin was then added to each well. ϵ -amino-n-caproic acid was also added (300 μ g/ml) for the first 4 days and twice weekly thereafter (50 μ g/ml) in order to prevent dissolution of the fibrin clot. The vessels were cultured at 37°C in a humidified incubator for 25 days with the media being changed twice weekly.

20 The degree of new vessel formation was quantified blind twice a week by three different observers using a visual analogue scale in which 0 = no growth, 1 = very little new vessel formation, 2 = significant new vessel formation and 3 = dense new vessel formation. An angiogenesis score was derived from the counts undertaken for each well by dividing the total score by the maximum score possible and then expressing the result as a percentage. 25 On 2 separate occasions, each compound was tested in quadruplicate replicate wells. Experiments in which blood vessels were cultured in MCDB 131 (i.e.: serum free media) showed that serum factors were not necessary for new blood vessel formation. Some of the fibrin clots were fixed overnight in 10% formalin, paraffin embedded, sectioned for histology and then stained for the endothelial cell markers Factor VIII and CD31.

30 Over the 28 day course of the assay, a complex arcade of microvessels emerged from the cut section of the blood vessel embedded within a fibrin clot. Very little growth of new vessels was seen from those areas of the vessel which had not been sectioned. The first blood vessels appeared by day 4 and were usually blunt-ended. Over the next 18 days, they went on to branch and give rise to complex arcades of vessels. Growth was rapid for

the first 2 weeks and then slowed considerably. These new vessels were lined by endothelial cells as shown by positive immunohistochemistry for Factor VIII and CD31.

In six experiments performed, there was a reduction in the number of new vessels that 5 were formed when dendrimer gen 3.5-glucosamine 6-sulfate was present at 25 μ g/ml (Figure 33). The inhibition of new vessel formation was more marked and significant (p<0.05) when the dendrimer gen 3.5-glucosamine 6-sulfate was present at 50 μ g/ml. This effect was first seen at day 18 with the difference persisting and becoming greater until day 28. The experiment was terminated on day 32.

10

EXAMPLE G: MODEL SYSTEM FOR A MIXED LYMPHOCYTE REACTION:

The present invention also provides a model system for the mixed lymphocyte reaction that can be used to evaluate compounds that inhibit the complex process of allograft organ rejection. The results from these experiments are shown in Figure 23 (i), Figure 23 15 (ii), Figure 23 (iii), Figure 24 (i), and Figure 24 (ii). Using our experimental model system, we have evaluated the anti-inflammatory activity of dendrimer gen. 3.5-glucosamine and by dendrimer gen. 3.5-glucosamine 6-sulfate. Our results show that the mixed lymphocyte reaction can be successfully inhibited by dendrimer gen. 3.5-glucosamine as well as by dendrimer gen. 3.5-glucosamine 6-sulfate, and that they could 20 therefore be of therapeutic use in preventing the inflammatory response seen when rejection of an allogeneic organ transplant occurs. It may also be effective in maintaining the counter inflammatory environment that is required to prevent the rejection of the transplanted organ, as well as the development of graft-versus-host disease.

25 The mixed lymphocyte reaction can be used to test the responsiveness of recipient lymphocytes to antigens expressed on donor cells. Low recipient anti-donor mixed lymphocyte reaction responses are associated with excellent transplant survival. The test is especially important in bone marrow transplantation, because it also assesses whether the donor bone marrow cells can respond to recipient antigens and cause graft-versus-host 30 disease.

Figure legends: (All results are expressed as the mean \pm sem.)

Figures 1a and 1b:

Structures of PAMAM anionic dendrimers gen 2.5 (a)

and PAMAM anionic dendrimers gen 3.5 (b).

Dendrimers gen 2.5: MW_t = 6,011 Daltons; 32 carboxylic surface groups.

Dendrimers gen 3.5: MW_t = 12,419 Daltons; 64 carboxylic surface groups.

5

Figure 2:

Schematic representation of the conjugation of glucosamine or sulfated glucosamine to PAMAM anionic dendrimers.

10 1 represents glucosamine (R= H, R₁=H, R₂=H) or glucosamine 6-sulfate (R=SO₃H, R₁=H, R₂=H) or glucosamine 3,6-disulfate (R=SO₃H, R₁= SO₃H, R₂=H) or glucosamine 3,4,6-trisulfate (R=SO₃H, R₁=SO₃H, R₂= SO₃H).

15 2 represents PAMAM anionic dendrimers gen 2.5 (x equal 32) or PAMAM anionic dendrimers gen 3.5 (x equal 64).

3 represents 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride used as the 20 condensing agent. y molecules of glucosamine or sulfated glucosamine bound to the dendrimers.

Figure 3:

Gel filtration of dendrimers gen 3.5-glucosamine mixture.

25 At the end of the reaction time 0.5 ml of the mixture (closed circles) was passed through a PD-10 column and the fractions collected read using a β -counter. Free glucosamine (open triangles), passed through a PD-10 column and the fractions collected read using a β -counter. Similar results were obtained for dendrimer gen 2.5-glucosamine.

25 Figure 4:

FT-IR spectra of dendrimers gen 3.5 (a),

glucosamine (b),

and dendrimers gen 3.5-glucosamine (c).

30 Figure 5:

Schematic representation of dendrimers gen 3.5-glucosamine.

Figure 6:

FT-IR spectra of dendrimers gen 3.5 (a),
glucosamine 6-sulfate (b),
and dendrimer gen 3.5-glucosamine 6-sulfate (c).

5 Figure 7(i):

Stability of the dendrimers gen 3.5-glucosamine during storage.

Dendrimers gen 3.5-glucosamine was stored as an aqueous solution. An aliquot of the aqueous solution was taken at day 0 (open triangles), day 7 (filled circles), day 15 (filled squares), day 90 (filled triangles) passed through a PD-10 column and the fractions collected read using a β -counter. Free glucosamine (open squares) passed through the PD-10 column and the fractions collected were read using a β -counter.

10 Figure 7(ii):

Stability of the dendrimers gen 2.5-glucosamine during storage.

15 Dendrimers gen 2.5-glucosamine was stored as an aqueous solution. An aliquot of the aqueous solution was taken at day 0 (filled triangles) and day 30 (filled circles), passed through a PD-10 column and the fractions collected read using a β -counter. Free glucosamine (open squares) passed through the PD-10 column and the fractions collected were read using a β -counter.

20

Figure 8(i):

No release of MIP-1 β above that seen in the control wells occurred with PBMN cells and dendrimer gen 3.5, dendrimer gen 3.5-glucosamine, or dendrimer gen 3.5-glucosamine 6-sulfate at 50 μ g/ml.

25

Figure 8(ii) and 8(iii):

No release of MIP-1 β above that seen in the control wells occurred with MDMs and dendrimer gen 3.5, dendrimer gen 3.5-glucosamine, or dendrimer gen 3.5-glucosamine 6-sulfate at 50 μ g/ml.

30

Figure 9:

No release of MIP-1 β above that seen in the control wells occurred with MDMs and dendrimer gen 3.5-glucosamine 3,6-disulfate, or dendrimer gen 3.5-glucosamine 3,4,6-trisulfate at 50 μ g/ml.

5 Figure 10(i) and (ii):

No release of MIP-1 β above that seen in the control wells occurred with peritoneal macrophages after 36 h of culture with dendrimer gen 3.5, dendrimer gen 3.5-glucosamine, or dendrimer gen 3.5-glucosamine 6-sulfate with each molecule at 25 μ g/ml.

10 Figure 10(iii):

No release of MIP-1 β above that seen in the control wells occurred with peritoneal macrophages after 72 h of culture with dendrimer gen 3.5, dendrimer gen 3.5-glucosamine, or dendrimer gen 3.5-glucosamine 6-sulfate with each molecule at 25 μ g/ml.

15 Figure 11(i) and (ii):

No release of MIP-1 β above that seen in the control wells occurred with peritoneal macrophages after 36 h of culture with dendrimer gen 3.5, dendrimer gen 3.5-glucosamine, or dendrimer gen 3.5-glucosamine 6-sulfate with each molecule at 50 μ g/ml.

20

Figure 12(i) and (ii):

No release of MIP-1 β above that seen in the control wells occurred with peritoneal macrophages after 36 h of culture with dendrimer gen 3.5-glucosamine 3,6-disulfate, or dendrimer gen 3.5-glucosamine 3,4,6-trisulfate with each molecule at 50 μ g/ml.

25

Figure 13(i) and (ii):

No release of MIP-1 β above that seen in the control wells occurred with peritoneal macrophages after 36 h of culture with dendrimer gen 3.5-glucosamine 3,6-disulfate, or dendrimer gen 3.5-glucosamine 3,4,6-trisulfate with each molecule at 100 μ g/ml.

30

Figure 13(iii) and (iv):

No release of MIP-1 β above that seen in the control wells occurred with peritoneal macrophages after 36 h of culture with dendrimer gen 3.5-glucosamine 3,6-disulfate, or dendrimer gen 3.5-glucosamine 3,4,6-trisulfate with each molecule at 200 μ g/ml.

5 Figure 14(i):

The dendrimer gen 3.5-glucosamine was cultured with MDMs for 72 h up to a concentration of 150 μ g/ml. No reduction in cell count or viability (as determined by Trypan blue and MTT assay) was found.

10 Figure 14(ii):

The dendrimer gen 3.5-glucosamine was cultured with HUVECs for 72 h up to a concentration of 100 μ g/ml. No reduction in cell count or viability (as determined by Trypan blue and MTT assay) was found.

15 Figure 15:

Single donor PBMN cells were isolated and cultured with LPS at 5 ng/ml. Cell free culture supernatants were harvested at regular intervals for up to 21 h and assayed for MIP-1 β .

Figure 16(i)

20 Single donor PBMN cells were cultured with dendrimer gen 3.5-glucosamine (100 μ g/ml) for 30 min and LPS was then added at 5 ng/ml. Cell free culture supernatants were harvested 21 h later and assayed for MIP-1 β . A significant reduction in MIP-1 β release was seen.

25 Figure 16(ii)

Single donor PBMN cells were cultured with dendrimer gen 3.5-glucosamine (100 μ g/ml) for 1 h and LPS was then added at 5 ng/ml. Cell free culture supernatants were harvested 21 h later and assayed for MIP-1 β . A significant reduction in MIP-1 β release was seen.

30 Figure 16(iii):

Single donor PBMN cells were cultured with dendrimer gen 3.5-glucosamine (100 μ g/ml) for 24 hours and LPS was then added at 5 ng/ml. Cell free culture supernatants were

harvested 21 h later and assayed for MIP-1 β . A significant reduction in MIP-1 β release was seen.

Figure 16(iv):

5 Single donor PBMN cells were cultured with dendrimer gen 3.5-glucosamine (100 μ g/ml) for periods ranging from 30 minutes or 1 hour or 3 hours or 6 hours or 24 hours and LPS was then added at 5 ng/ml. Cell free culture supernatants were harvested 21 h later and assayed for MIP-1 β . A significant reduction in MIP-1 β release was seen at all of the time points studied.

10

Figure 17:

Single donor PBMN cells were isolated and cultured with LPS at 5 ng/ml. Cell free culture supernatants were harvested at regular intervals for up to 21 h and assayed for TNF- α .

15 Figures 18(i) to (vi):

Single donor PBMN cells were cultured with dendrimer gen 3.5-glucosamine (100 μ g/ml) for 30 min and LPS was then added at 5 ng/ml. Cell free culture supernatants were harvested 21 h later and assayed for MIP-1 α as shown in Figure 18(i), MIP-1 β as shown in Figure 18(ii), IL-8 as shown in Figure 18(iii), TNF- α as shown in Figure 18(iv), IL-1 β 20 as shown in Figure 18(v), and IL-6 as shown in Figure 18(vi). For all of the chemokines and pro-inflammatory cytokines studied, a significant reduction in their release was seen when dendrimer gen 3.5-glucosamine was present.

Figures 19(i) to (vi):

25 Single donor PBMN cells were cultured with dendrimer gen 3.5-glucosamine (200 μ g/ml) and LPS was then added at 5 ng/ml. Cell free culture supernatants were harvested 21 h later and assayed for MIP-1 α as shown in Figure 19(i), MIP-1 β as shown in Figure 19(ii), IL-8 as shown in Figure 19(iii), TNF- α as shown in Figure 19(iv), IL-1 β as shown in Figure 19(v), and IL-6 as shown in Figure 19(vi). For all of the chemokines and pro-inflammatory cytokines studied, a significant reduction in their release was seen when 30 dendrimer gen 3.5-glucosamine was present.

Figures 20(i) and (ii):

Single donor PBMN cells were cultured with dendrimer gen 3.5-glucosamine (200 µg/ml) for 1 hour and LPS was then added at 5 ng/ml. Cell free culture supernatants were harvested 25 h later and assayed for MIP-1 β as shown in Figure 20(i) and TNF- α as shown in Figure 20(ii). For all of the chemokines and pro-inflammatory cytokines studied, a significant reduction in their release was seen when dendrimer gen 3.5-glucosamine was present.

Figure 21:

Single donor PBMN cells were isolated and resuspended in RPMI, L-glutamine, penicillin, streptomycin and 10% human serum at a density of 1×10^6 cells/ml. The cells were then plated (1 ml) in 12 well tissue culture plates and cultured for 15 min at 37°C in 5% CO₂. LPS was then added at 5 ng/ml and the cells were cultured for 30 min, or 1 hour, or 2 hours, or 3 hours, or 4 hours at 37°C in 5% CO₂ before adding dendrimer gen 3.5-glucosamine at 100 µg/ml. Cell free culture supernatants were harvested 21 h after the addition of the LPS and assayed for MIP-1 α as shown in Figure 21(i), MIP-1 β as shown in Figure 21(ii), IL-8 as shown in Figure 21(iii), TNF- α as shown in Figure 21(iv), IL-1 β as shown in Figure 21(v), and IL-6 as shown in Figure 21(vi). For all of the chemokines and pro-inflammatory cytokines studied, a significant reduction in their release was seen when dendrimer gen 3.5-glucosamine was present.

20

Figure 22:

Single donor PBMN cells were cultured with dendrimer gen 3.5-glucosamine in which the loading of glucosamine was 3% (rather than the 7% used in all other experiments) at a concentration of 150 µg/ml or 300 µg/ml. The cells were cultured for 30 min at 37°C in 5% CO₂ and LPS was then added at 5 ng/ml. Cell free culture supernatants were harvested 22 h later and assayed for MIP-1 β as shown in Figure 22(i), or for TNF- α as shown in Figure 22(ii). For all of the chemokines and pro-inflammatory cytokines studied, a significant reduction in their release was seen when dendrimer gen 3.5-glucosamine was present.

Figure 23:

Single donor PBMN cells were isolated from 3 individuals and mixed together for 72 h. Dendrimer gen 3.5-glucosamine was then added at 50 - 100 µg/ml. Cell free culture supernatants were harvested 1 day, 3 days and 4 days later and assayed for MIP-1 β and

TNF- α . A significant reduction in MIP-1 β and TNF- α release into the culture supernatant was seen at all 3 time points assayed as shown in Figure 23(i) and 23 (ii).

5 Single donor PBMN cells were isolated from 2 individuals and then cultured separately for 24 h. They were then mixed together for 72 h. The dendrimer gen 3.5-glucosamine was then added at 50 μ g/ml. Cell free culture supernatants were harvested 1 day, 3 days and 4 days later and assayed for MIP-1 β . A significant reduction in MIP-1 β release into the culture supernatant was seen at all 3 time points assayed as shown in Figure 23(iii).

10 Figure 24:

Single donor PBMN cells were isolated from 2 individuals and mixed together. Dendrimer gen 3.5-glucosamine was then added at 100 μ g/ml followed immediately by LPS (10 ng/ml). Cell free culture supernatants were harvested at regular intervals for up to 50 h. A significant reduction in MIP-1 β release into the culture supernatant was seen from 24 h 15 onwards as shown in Figure 24(i).

20 Single donor PBMN cells were isolated from 2 individuals and mixed together. Dendrimer gen 3.5-glucosamine was added at 100 μ g/ml. Twenty-four hours later, LPS was added at a concentration of 5 ng/ml. Cell free culture supernatants were harvested at regular intervals for up to 100 h. A significant reduction in MIP-1 β release into the culture supernatant was seen from 24 h onwards as shown in Figure 24(ii).

Figure 25:

25 The dendrimer gen 3.5-glucosamine 6-sulfate did not affect the cell viability or the growth characteristics of PBMN cells when present at a concentration of 50 μ g/ml or 150 μ g/ml in cultures maintained for up to 5 days. Cell viability was determined by Trypan blue exclusion and an MTT assay.

Figure 26:

30 The dendrimer gen 3.5-glucosamine 6-sulfate did not affect cell viability or growth characteristics of MDMs when present up to a concentration of 150 μ g/ml in cultures maintained for up to 5 days. Cell viability was determined by Trypan blue exclusion and an MTT assay.

Figure 27 (i) and (ii) & (iii):

The dendrimer gen 3.5-glucosamine 6-sulfate construct was also not toxic to HUVECs when added to cultures of these cells up to a concentration of 100 μ g/ml for up to 72 hours.

Figure 28:

PBMN cells were cultured with dendrimer gen 3.5-glucosamine 6-sulfate (150 μ g/ml or 200 μ g/ml) for 30 min and LPS (5 ng/ml) was then added. Cell free culture supernatants were harvested 24 h later for MIP-1 β (Figure 28(i)) and for TNF- α (Figure 28(ii)). A significant reduction in both the chemokine and the pro-inflammatory cytokine was seen when dendrimer gen 3.5-glucosamine 6-sulfate was present at 150 μ g/ml and at 200 μ g/ml.

Figure 29:

PBMN cells were isolated from 4 individuals and then mixed together for 24 h. MDMs were separated by adherence to plastic and then cultured with dendrimer gen 3.5-glucosamine or dendrimer gen 3.5-glucosamine 6-sulfate at 125 μ g/ml. Cell free culture supernatants were harvested 36 h later and assayed for MIP-1 β . A significant reduction in MIP-1 β was seen.

Figures 30 - 32:

Endothelial microtubule formation by HUVECs on Matrigel ($\times 40$ magnification). A visual analogue scale was used to determine the extent of tube formation and scored on a scale from 0 (all cells remain single) to 4 (all cells involved in tubular structures) as previously described (Bauer J, Margolis M, Schreiner C, Edgell C-J, Azizkhan J, Lazarowski E, & Juliano RL. (1992) In vitro model of angiogenesis using a human endothelium derived permanent cell line: contributions of induced gene expression, G-proteins and integrins. *J. Cellular Physiology* 153: 437-449; Liaw, L & Schwartz SM. (1993) Microtubule disruption stimulates DNA synthesis in bovine endothelial cells and potentiates cellular response to basic fibroblast growth factor. *American Journal of Pathology* 143: 937-948).

Figure 30: Control well,

Figure 31: Dendrimer gen 3.5-glucosamine 6-sulfate at 12.5 μ g/ml,

Figure 32: Dendrimer gen 3.5-glucosamine 6-sulfate at 50 μ g/ml.

5 **Figure 33:**

Dendrimer gen 3.5-glucosamine 6-sulfate (O) reduced the rate of new vessel formation in an in vitro angiogenesis assay when compared to media alone (Δ). The results shown are for dendrimer gen 3.5-glucosamine 6-sulfate at a final concentration of 50 μ g/ml. The degree of new vessel formation was quantified blind twice a week using a visual analog scale in which 0 = no growth, 1 = minimal new vessel formation, 2 = significant new vessel formation, and 3 = dense new vessel formation. An angiogenesis score was derived from each count by dividing the total score (i.e. the sum of all the wells) by the maximum possible score and then expressing the result as a percentage. A significant reduction in new vessel formation was seen by day 18 ($p<0.05$). This graph is representative of 2 experiments performed in quadruplicate. New vessel formation was quantified blind by 3 different observers twice a week with a within-observer and between-observer variability of <10%.

10 **Figure 34:**

20 Cartoon describing the pathogenesis of medical disorders in which chemokines and angiogenesis play an important role. It identifies the new targets at which dendrimer gen 3.5-glucosamine and dendrimer gen 3.5-glucosamine 6-sulfate can act either alone or in synergy to produce a clinically useful therapeutic effect.

Table 1:

This table shows the reproducibility of the synthesis of the dendrimer gen 3.5-glucosamine conjugate with a 7 % loading of glucosamine that was used for most of the experiments described. Results are also shown for the dendrimer gen 3.5-glucosamine conjugate with a 3 % loading of glucosamine that was used for Experiment B 12, Figure 22.

	mmol glucosamine used in reaction	% converted carboxylic groups	Unbound glucosamine content (wt %)	Yield (%)
3 % glucosamine loading	0.25	3.18 ± 0.46	2.3	63
7 % glucosamine loading	0.5	7.89 ± 0.27	3.1	60
7 % glucosamine loading	0.5	7.24 ± 0.51	2.8	60

The total amount of glucosamine bound to the dendrimers was determined using a β -counter (data shown as mean \pm SD of 3 replicates). The unbound glucosamine content was determined using a β -counter followed by gel filtration (PD-10 column) of the purified conjugate.

Table 2:
Characterisation of the different batches of dendrimer gen 3.5-sulfated glucosamine conjugates prepared.

	Sulphur content (% m:m S)	Yield (%)
3.5-G6S (batch 1)	1.76	53
3.5-G6S (batch 2)	2.79	85
3.5-G3,6S (batch 1)	5.18	70
3.5-G3,6S (batch 2)	5.78	68
3.5-G3,4,6S (batch 1)	7.65	81
3.5-G3,4,6S (batch 2)	7.93	70

⁵ The amount of sulfated sugar bound to the dendrimer gen 3.5 was determined by sulphur analysis.

'3.5-G6S' stands for dendrimers gen 3.5-glucosamine 6-sulfate,

'3.5-G3,6S' stands for dendrimer gen 3.5-glucosamine 3,6-disulfate,

'3.5-G3,4,6S' stands for dendrimer gen 3.5-glucosamine 3,4,6-trisulfate.

5 **Table 3:**
 Toxicity of dendrimers gen 3.5-glucosamine and dendrimers gen 3.5-glucosamine 6-sulfate conjugates in several human cell lines and in a
 murine melanoma cell line.

	Sup-T1	C8166	U937	U87-CD4-CCR5	B16F10
DG 3.5-G	1825 ± 227	3000 ± 100	2837 ± 107	2077 ± 237	2894 ± 96
DG 3.5-G6S	307 ± 29	279 ± 7	271 ± 19	256 ± 21	246 ± 17
Dendrimers gen 3.5	> 5000	> 5000	> 5000	> 5000	> 5000
G	> 1000	> 1000	> 1000	> 1000	> 1000
G6S	> 1000	> 1000	> 1000	> 1000	> 1000
Dextran	> 5000	> 5000	> 5000	> 5000	> 5000
Poly(L-lysine)	25 ± 1	28 ± 1	28 ± 1	28 ± 0	28 ± 1

5 The toxicity of the conjugates and reference controls was analysed against the human cell lines Sup-T1, C8166, U937, and U87-CD4-CCRS,
 and the murine melanoma cell line (B16F10).

10 The IC₅₀ values (μg/ml) ± SEM (n = 16) were determined using the MTT assay.

15 'DG 3.5-G' stands for dendrimer gen 3.5-glucosamine.

'DG 3.5-G6S' stands for dendrimer gen 3.5-glucosamine 6-sulfate.

'G' stands for glucosamine.

'G6S' stands for glucosamine 6-sulfate.

Table 4a: Interaction of chemokines and cytokines with dendrimers gen 3.5-glucosamine conjugates:

	Standard concentration (pg/ml)	Standard Reading (pg/ml)	Standard + Dendrimer gen 3.5-glucosamine (pg/ml)
MIP-1 α	125	99.86	103.94
MIP-1 β	125	112.17	100.36
TNF- α	125	123.44	63.77
IL-1 β	31.2	28.23	23.80
IL-6	25	26.17	21.69
IL-8	250	298.30	280.15

There was no interference of the dendrimer gen 3.5 glucosamine with the MIP-1 α , MIP-1 β , IL-1 β , IL-6 or IL-8 assays.

Table 4b: Interaction of TNF- α with dendrimers gen 3.5-glucosamine conjugates:

In the case of TNF- α assay, a significant degree of interference by dendrimer gen 3.5 glucosamine was seen as illustrated below.

TNF- α standard (pg/ml)	TNF- α standard (EIA optical density)	TNF- α standard + dendrimer gen 3.5 glucosamine (EIA optical density)
15.60	0.062	0.044
31.25	0.096	0.058
62.50	0.169	0.090
125.00	0.273	0.161
250.00	0.514	0.326
500.00	0.935	0.596

A correction factor has therefore been applied to all of the TNF- α data shown as it relates to dendrimer gen 3.5 glucosamine.

Table 5: Anticoagulant activity of dendrimer gen 3.5-glucosamine and dendrimer gen 3.5-sulfated glucosamine conjugates:

The compounds were dissolved in phosphate-buffered saline solution (PBS) and tested, or dissolved in RPMI + 15% FCS and tested, or mixed 50 : 50 with human plasma/veronal buffer and tested. The anti-Xa activity was expressed as units of heparin/ml (HEP (Xa) (U/ml)).

		dissolved in	Conc (μ g/ml)	PT (sec)	APTT (sec)	TT (sec)	Fibrinogen (g/L)	Anti-Xa (U/ml)
Control	PBS		16.0	40	13.5	1.30	0	0
	RPMI/FCS		16.7	40	13.8	1.30	0	0
	Plasma/veronal buffer		16.5	41	13.7	1.30	0	0
D gen. 3.5-G	PBS	200	15.8	42	13.8	1.29	0	0
	RPMI/FCS	200	15.5	41	13.5	1.30	0	0
	Plasma/veronal buffer	300	15.6	42	13.7	1.31	0	0
D gen. 3.5-G6S	PBS	50	15.8	43	14.0	1.33	0	0
	RPMI/FCS	50	16.0	42	13.8	1.30	0	0
D gen. 3.5-G3,6S	PBS	200	15.8	43	14.2	1.36	0	0

Table 6: Endothelial microtubule formation in the presence of sulphated dextrans:

Compound	Tube formation score (n = 4)
control	4
dendrimers gen 3.5 (100 µg/ml)	4
glucosamine (100 µg/ml)	4
glucosamine 6-sulfate (100 µg/ml)	4
glucosamine 3,6-disulfate (100 µg/ml)	4
dendrimer gen 3.5-glucosamine 6-sulfate	
0 µg/ml	4
0.25 µg/ml	4
0.5 µg/ml	4
0.8 µg/ml	3
1.6 µg/ml	3
3.125 µg/ml	3
6.25 µg/ml	3
12 µg/ml	2
25 µg/ml	2
50 µg/ml	1
100 µg/ml	0
dendrimer gen 3.5-glucosamine 3,6-disulfate	
0 µg/ml	4
6.25 µg/ml	4
12 µg/ml	4
25 µg/ml	4
50 µg/ml	3
100 µg/ml	2

Statement as to US Government sponsored research:

This invention was made, in part, with US Government funds from the National Institutes of Health (grant number: 1-R21-A144694-01). The US Government has certain rights in the invention.

CLAIMS:

1. A process to covalently link biologically active or non-biologically active molecules to anionic dendrimers wherein the dendrimers are reacted with the molecule in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride.
5
2. A process according to claim 1 which is carried out at room temperature.
3. A process according to claim 1 or 2 wherein the covalent bond is synthesised in a
10 single step chemical reaction.
4. A process according to any one of claims 1 to 3 which is carried out in aqueous solution.
- 15 5. A process according to any one of claims 1 to 4 wherein the molecule is a drug, polynucleotide, protein, peptide, carbohydrate, antibody or lipid.
6. A process according to any preceding claim wherein the molecule is a saccharide with an amine group.
20
7. A process according to claim 6 wherein the amine group is a primary amine group.
8. A process according to claim 6 or 7 wherein the saccharide is glucosamine or sulfated glucosamine.
25
9. A process according to claim 8 wherein the sulphated glucosamine is D-glucosamine 6 sulphate, D-glucosamine 3,6-disulphate or D-glucosamine 3,4,6 trisulphate, D-glucosamine 3-sulphate, D-glucosamine 4-sulphate, or D-glucosamine 3,4 disulphate, D-glucosamine 4,6 disulphate.
- 30 10. An anionic glycodendrimer comprising covalently linked carboxylic terminated dendrimers.

11. An anionic glycodendrimer according to claim 10 wherein the dendrimers are PAMAM dendrimers.
12. An anionic glycodendrimer according to claim 11 wherein the dendrimers are PAMAM dendrimer generation 2.5.
13. An anionic glycodendrimer according to claim 11 wherein the dendrimers are PAMAM dendrimer generation 3.5.
14. An anionic glycodendrimer according to anyone of claims 10 to 13 wherein PAMAM anionic dendrimers are conjugated to molecules containing aminic groups or sulphated molecules containing aminic groups.
15. An anionic glycodendrimer according to claim 14 wherein the molecules are saccharides.
16. An anionic glycodendrimer according to anyone of claims 12 to 14 wherein PAMAM anionic dendrimers are conjugated to 3-amino-1-propanesulfonic acid.
17. An anionic glycodendrimer which is dendrimer gen. 3.5-glucosamine or dendrimer gen. 3.5-glucosamine 6-sulphate.
18. Anionic glycodendrimers according to any one of claims 10 to 17 for use in medicine.
19. Use of anionic glycodendrimers according to any one of claims 10 to 17 in the manufacture of a medicament for the treatment of diseases where chemokines and cytokines are increased.
20. Use of anionic glycodendrimers according to any one of claims 10 to 17 in the manufacture of a medicament for the treatment of diseases where angiogenesis is increased.

28. Pharmaceutical formulations according to claim 25 or 26 wherein the concentration of the glycodendrimer is 25 to 250 μ g/ml.

29. A method of treating a patient for severe sepsis or rheumatological disease by use of
5 an anionic glycodendrimer according to any one of claims 10 to 17.

Abstract:

The present invention relates to new anionic glycodendrimers having new biological activity, processes for preparing them and their use in medicine including veterinary medicine.

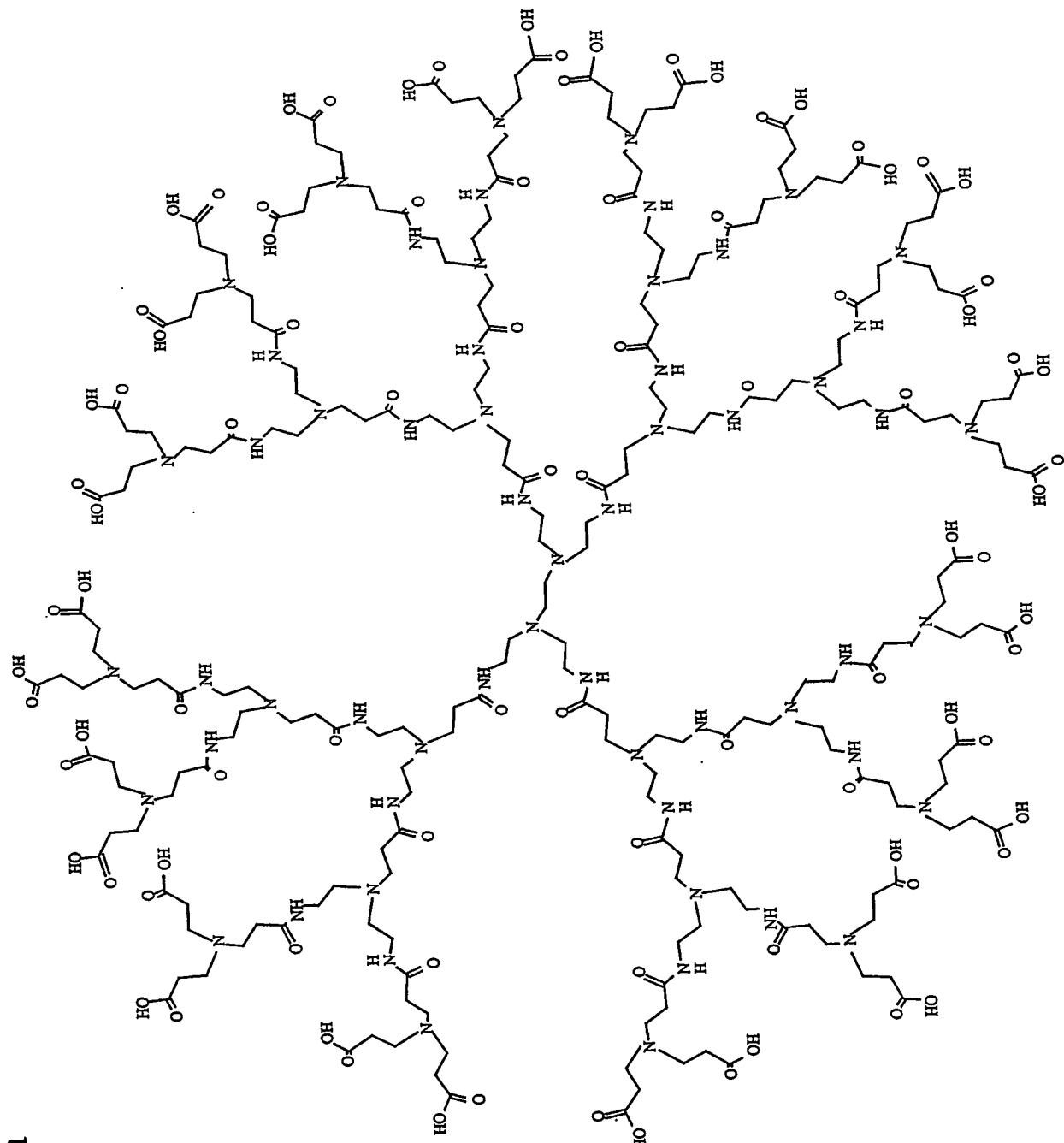


Figure 1a

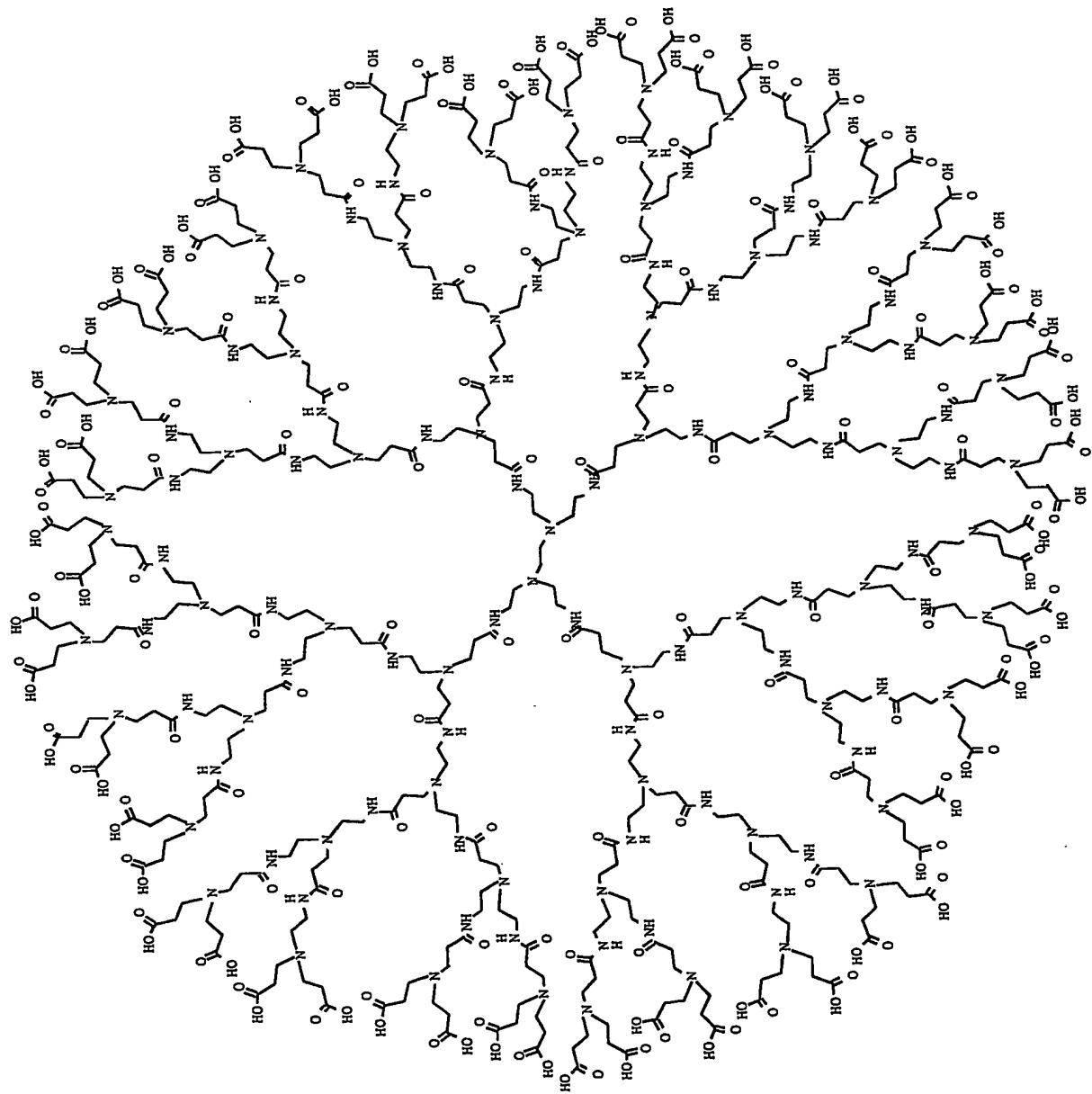


Figure 1b

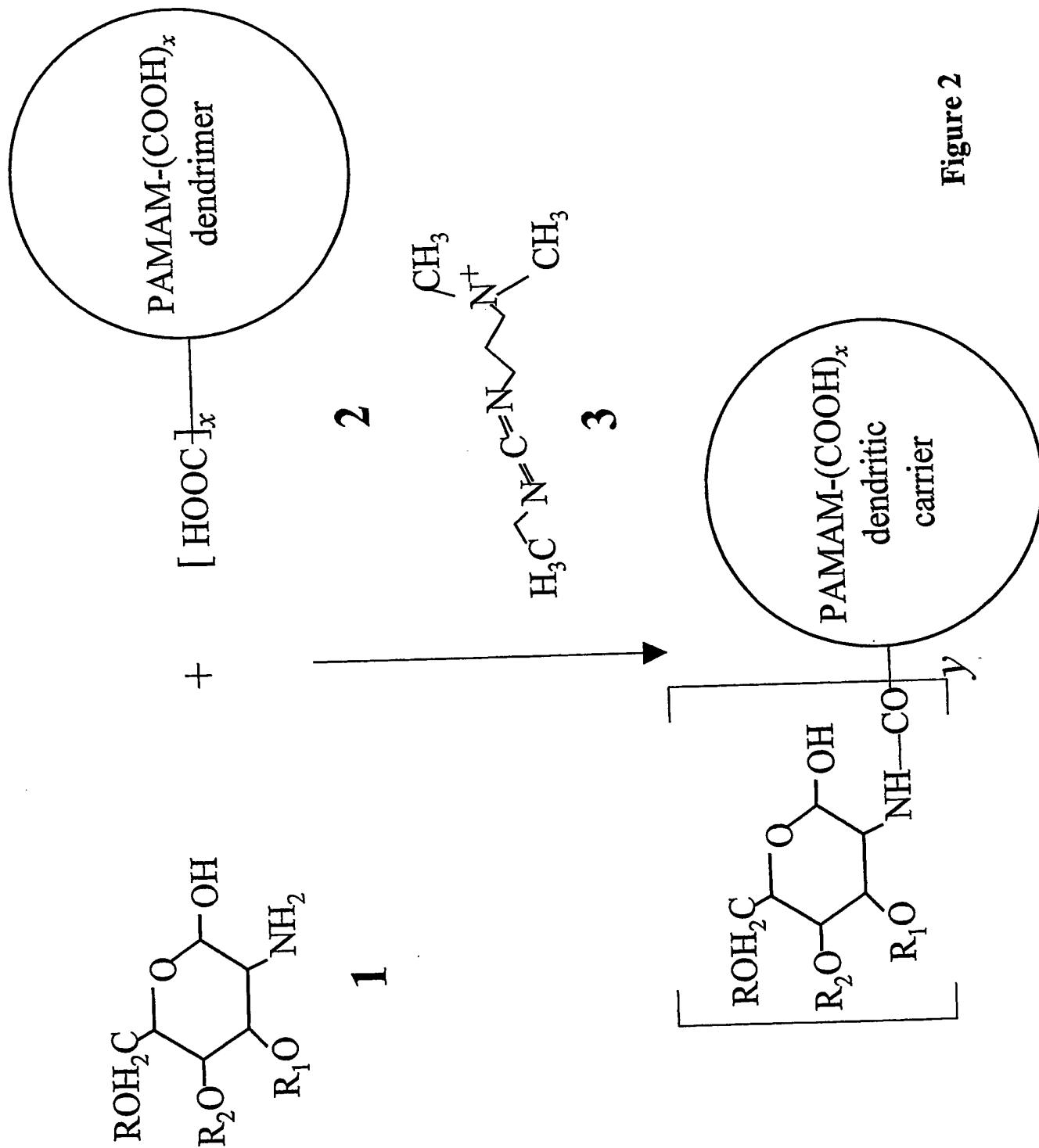
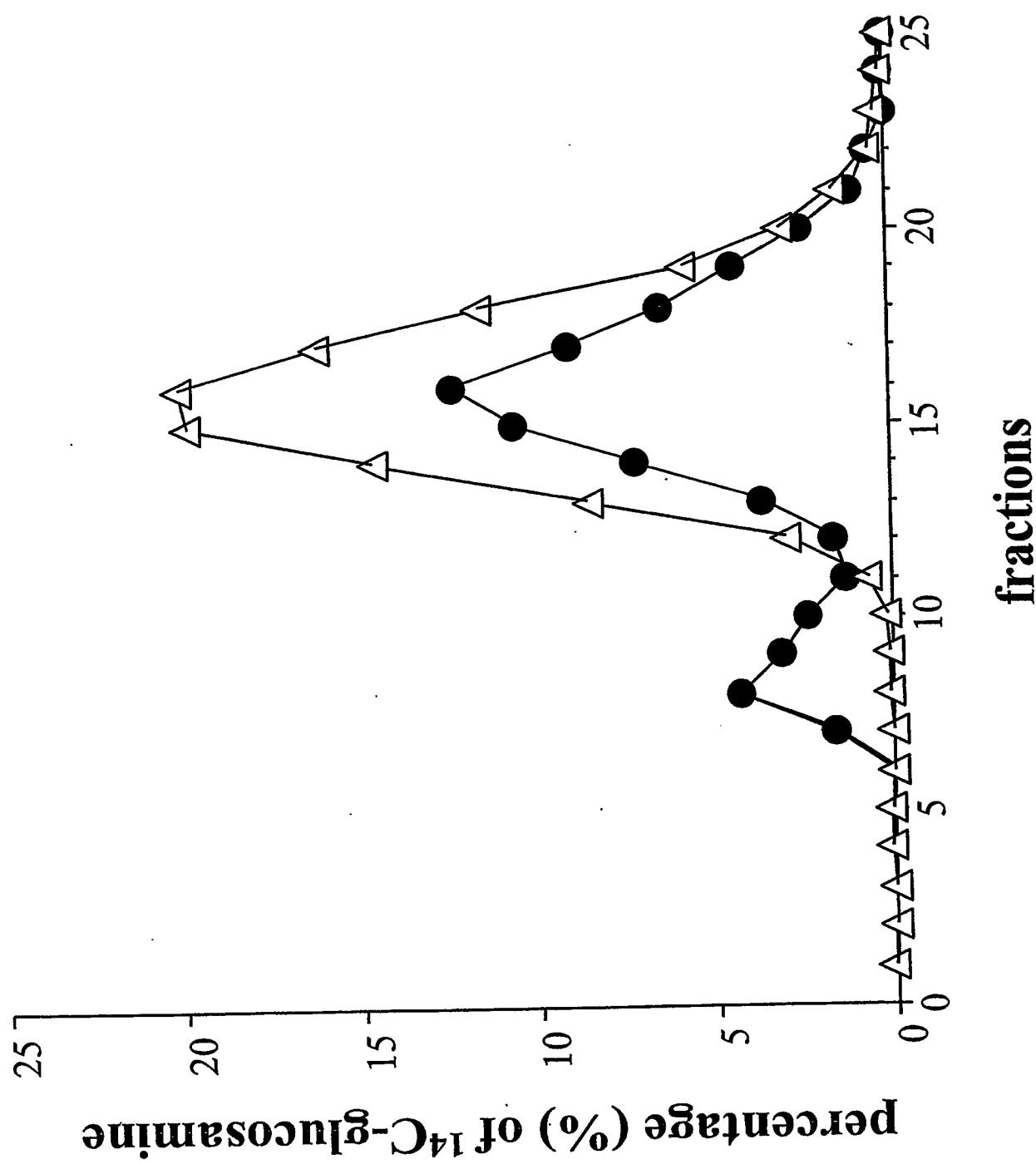


Figure 2

Figure 3



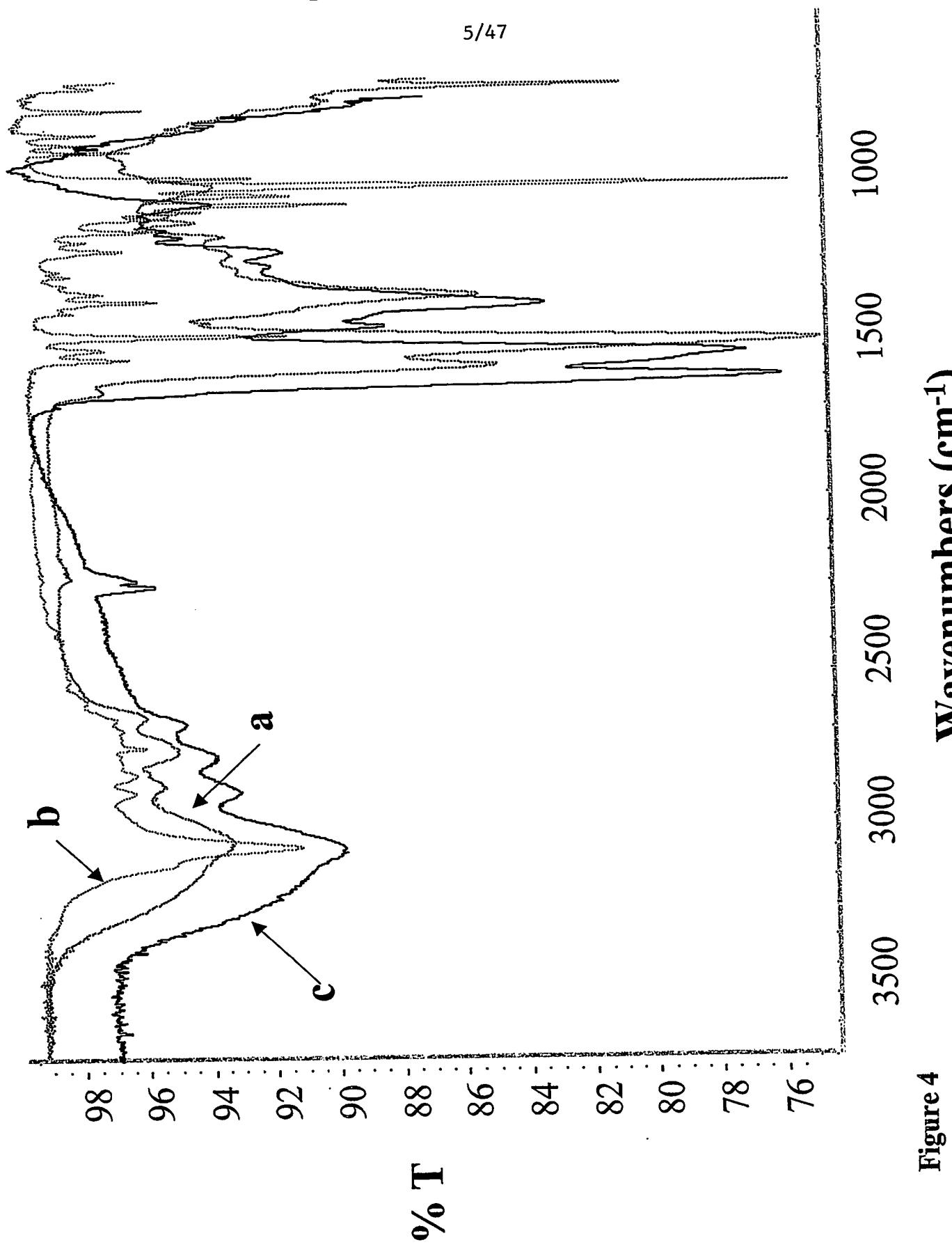
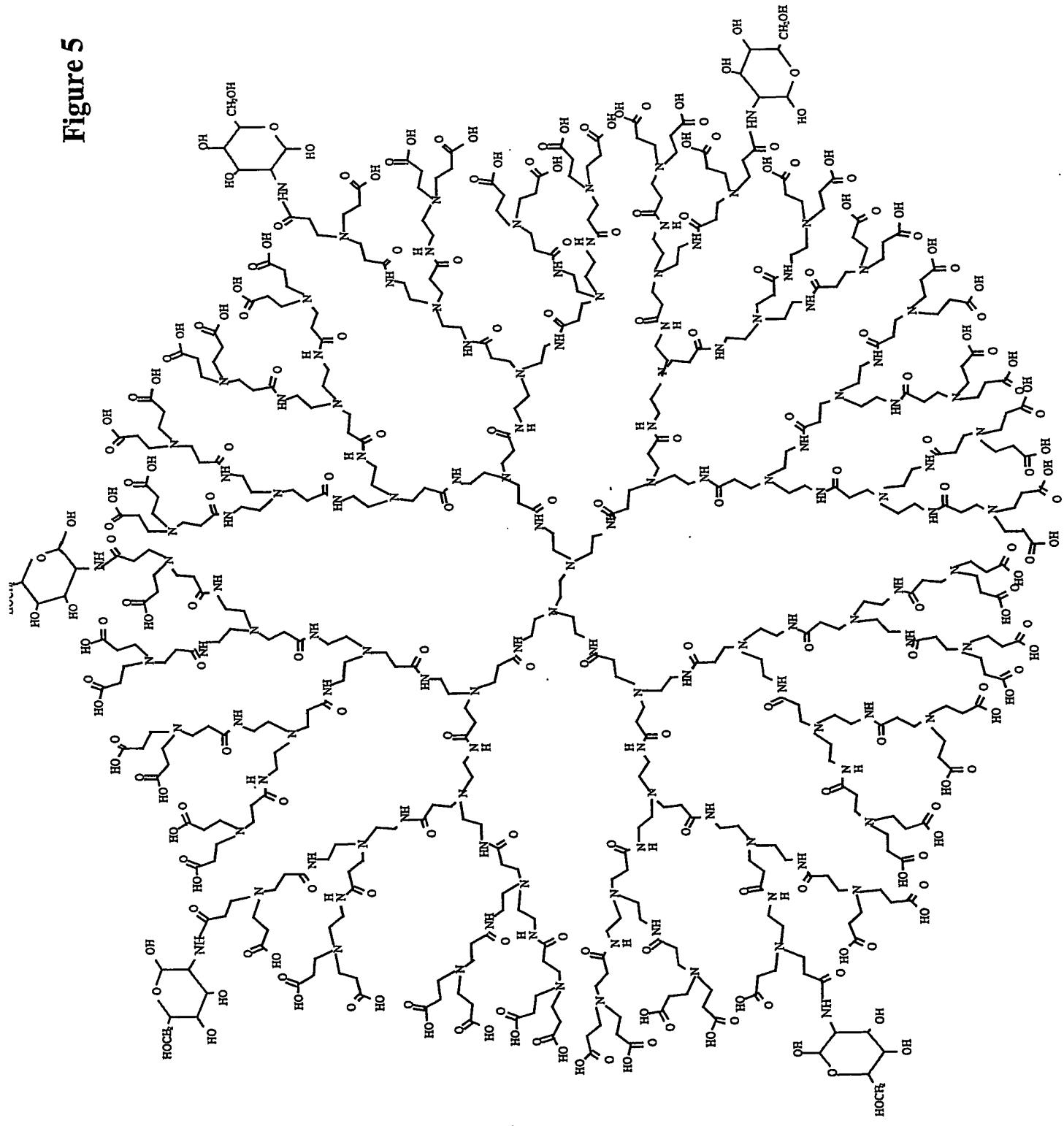


Figure 4

Wavenumbers (cm^{-1})

Figure 5



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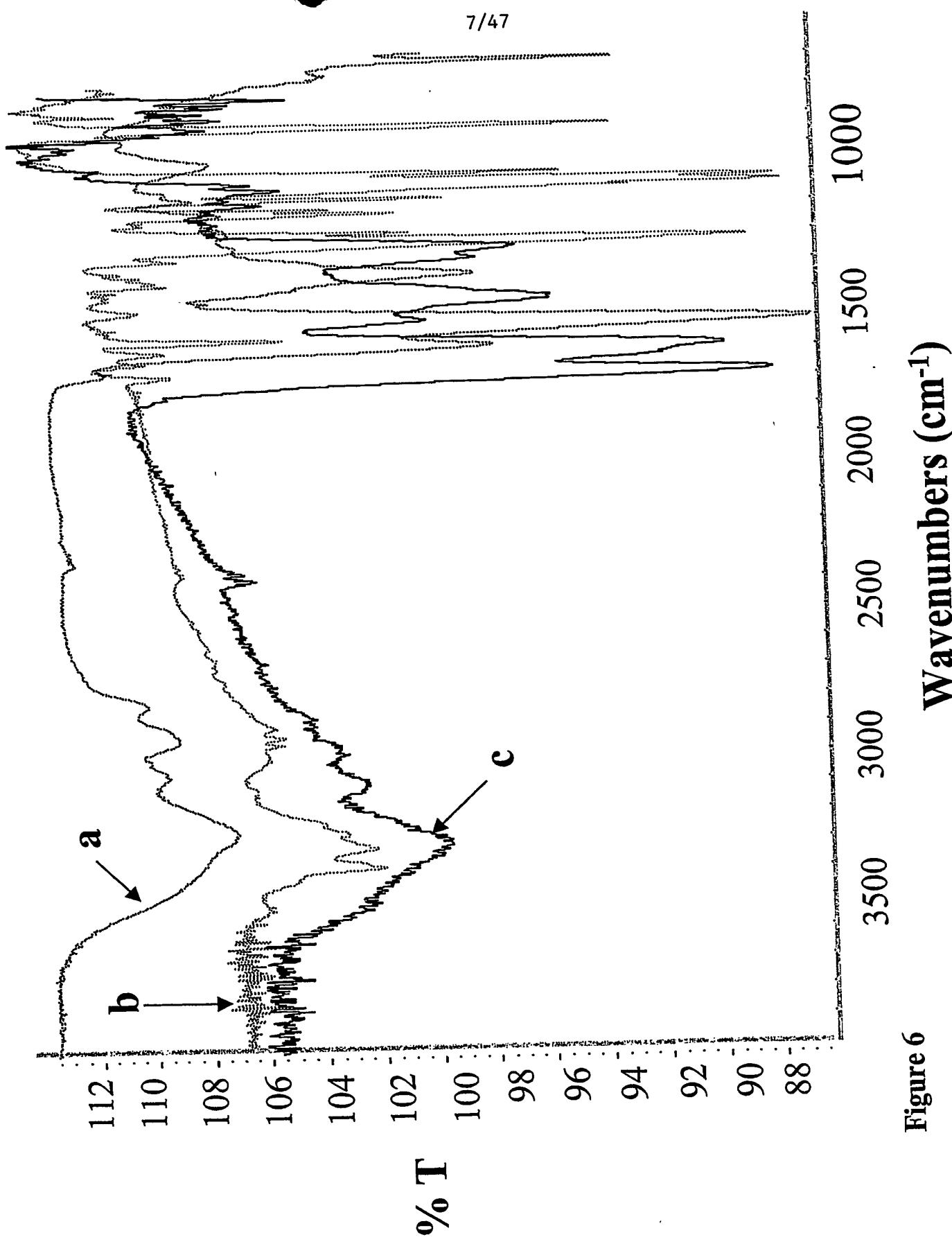
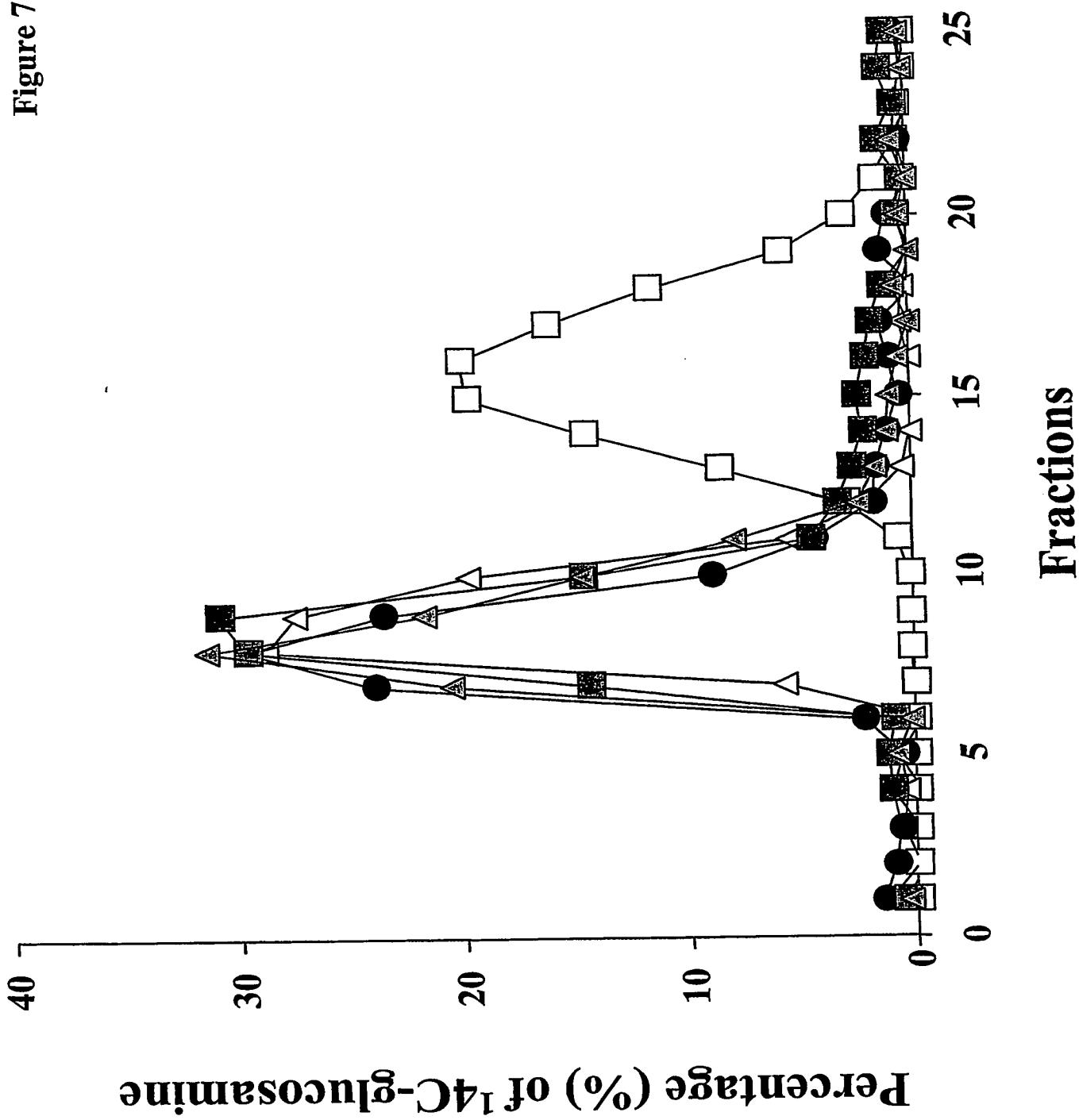


Figure 6

Wavenumbers (cm^{-1})

Figure 7(i)



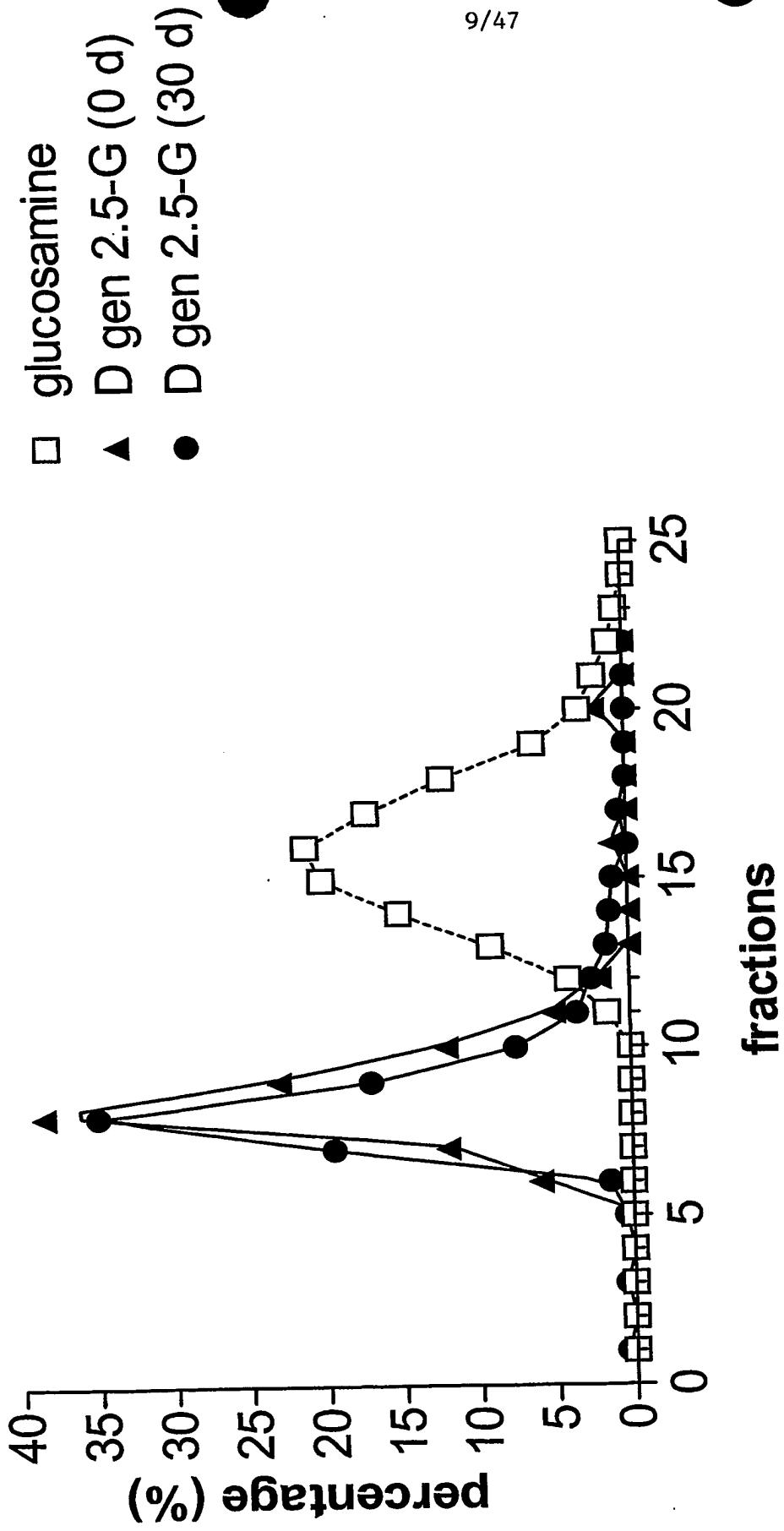
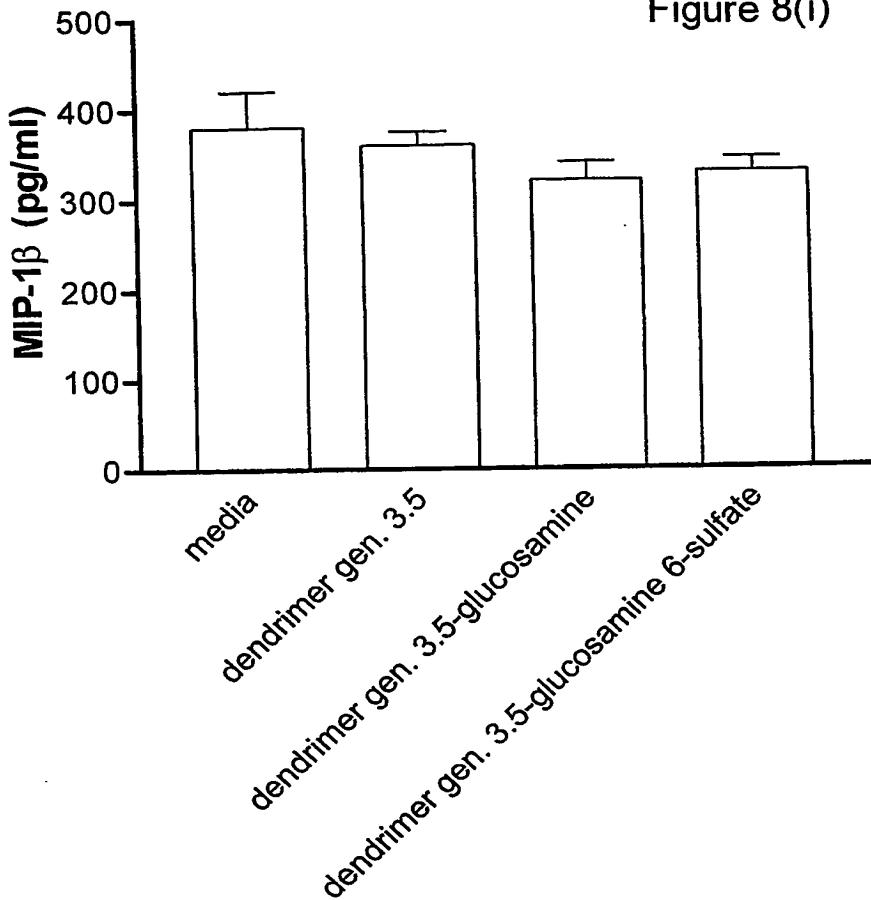


Figure 7(ii):
PD-10 column of dendrimers gen 2.5-glucosamine after synthesis [D gen 2.5-G (0 d)] and after 30 days of storage [D gen 2.5-G (30 d)]

Figure 8(i)



- n = 3. Results shown as mean \pm sem. p = ns.
- single donor PBMN cells.
- all compounds used at 50 μ g/ml.
- culture supernatants harvested for MIP-1 β at 36 h.

Figure 8(ii)

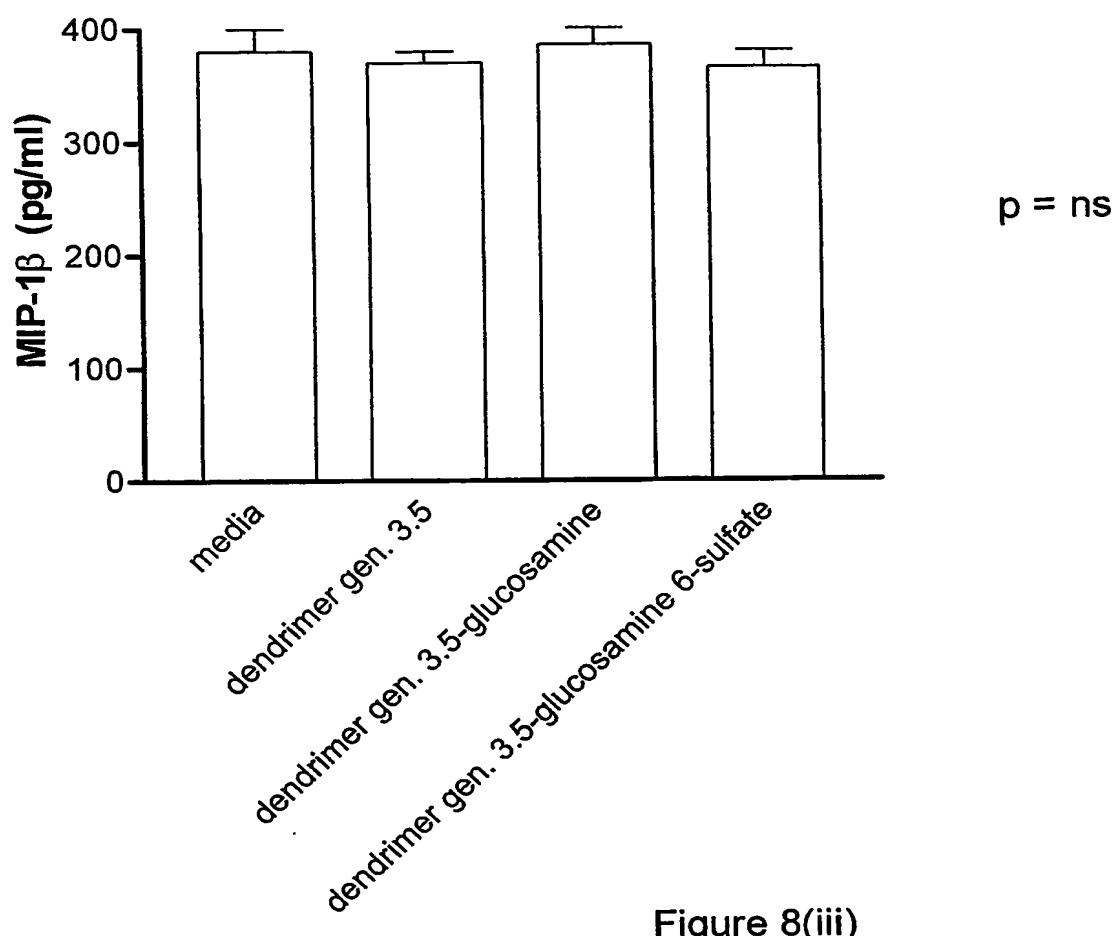


Figure 8(iii)

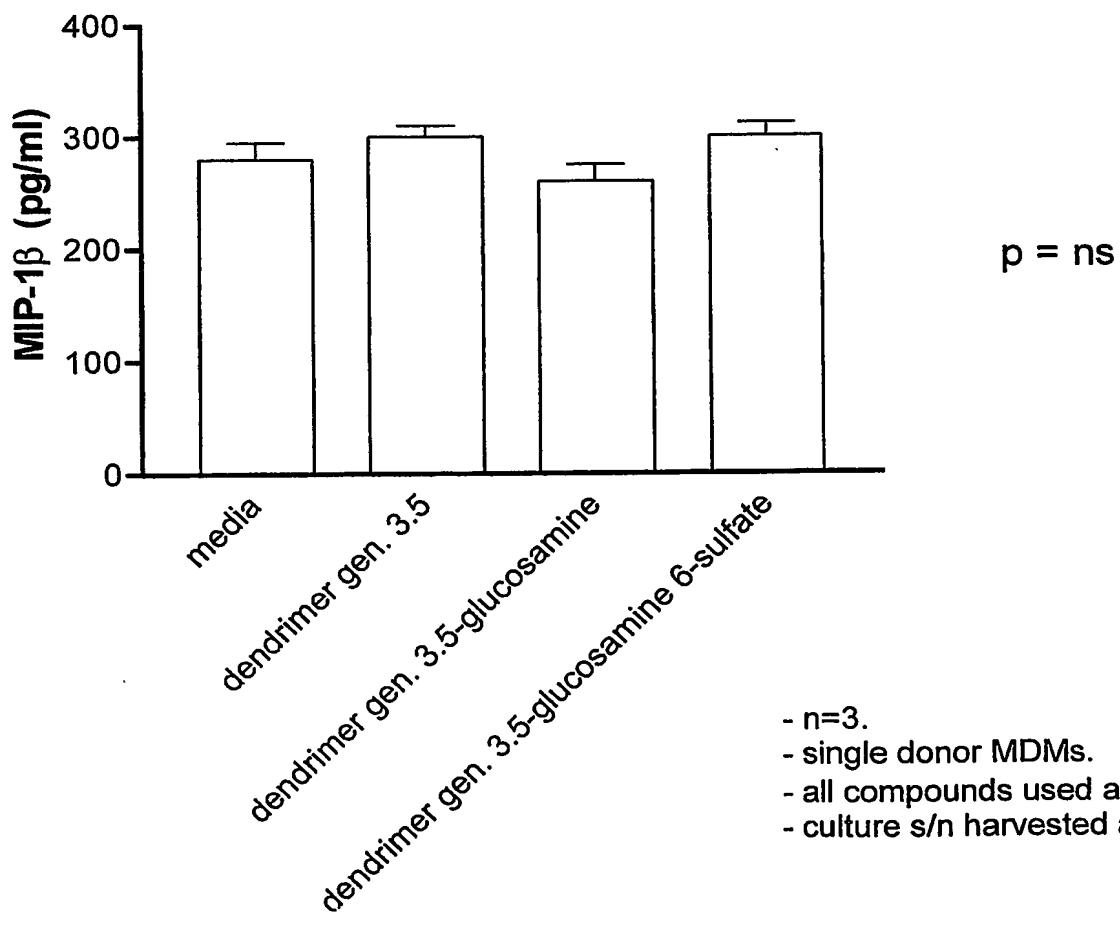
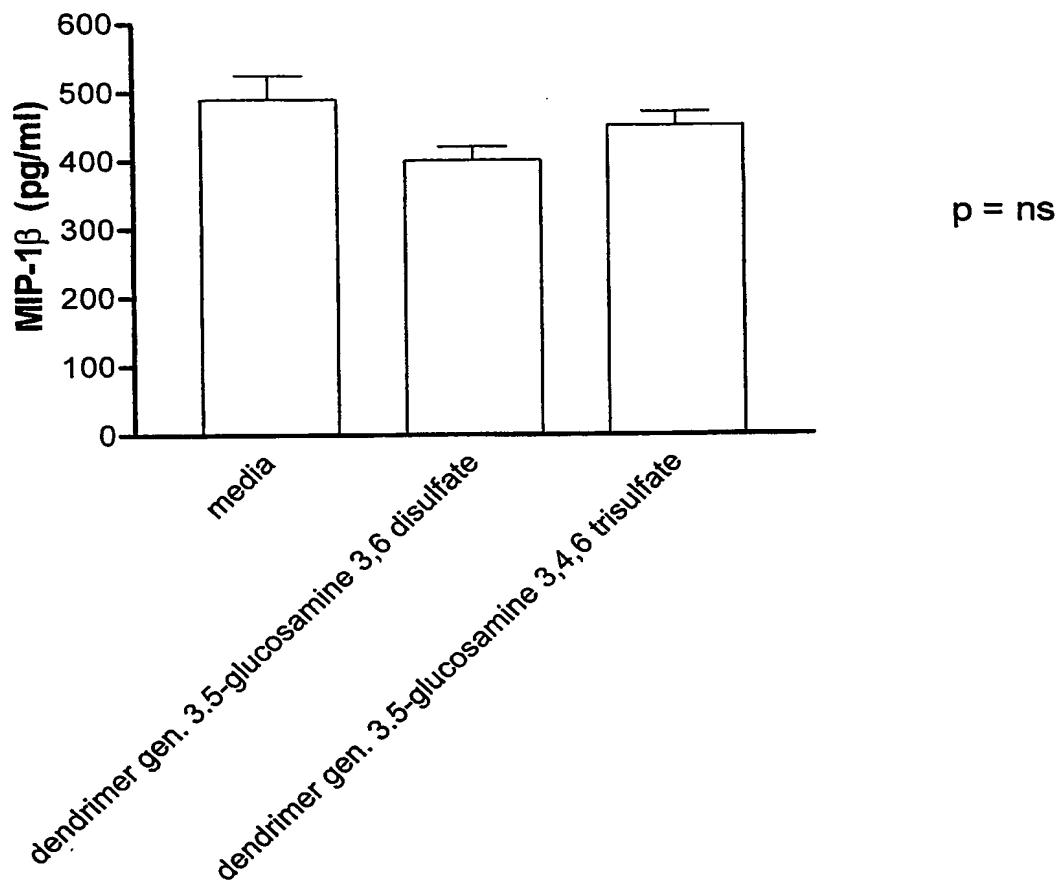


Figure 9



- n = 3.
- single donor MDMs.
- all compounds used at 50 μ g/ml.
- culture supernatants harvested for MIP-1 β after 36 h.

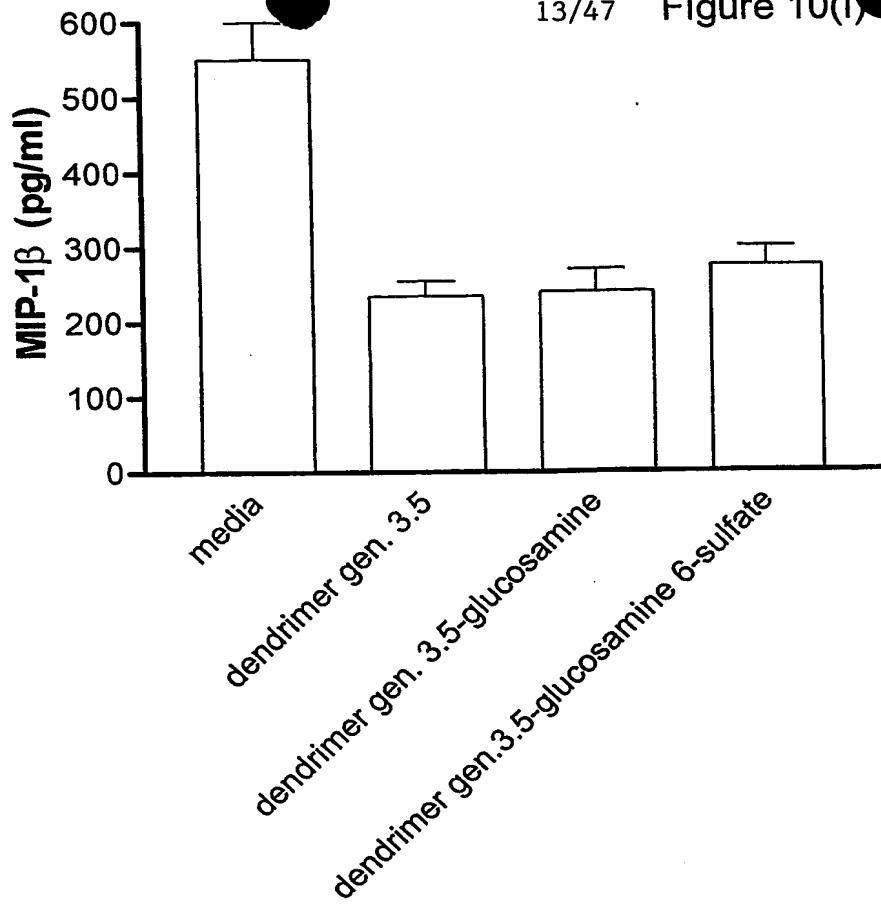
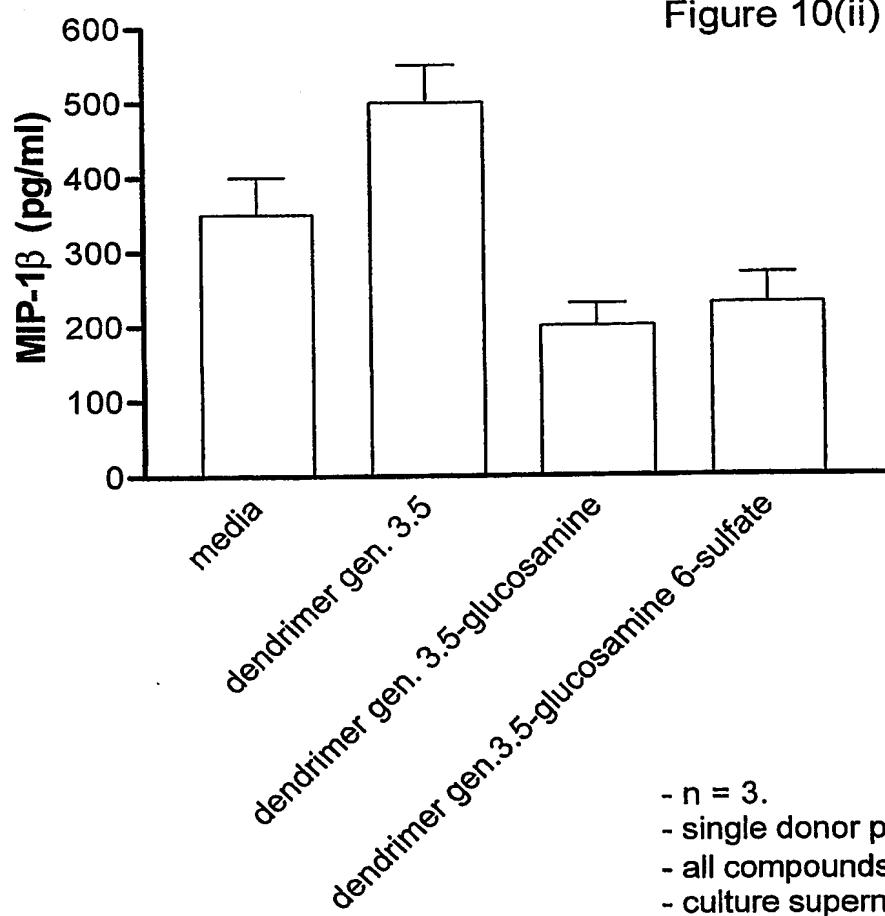


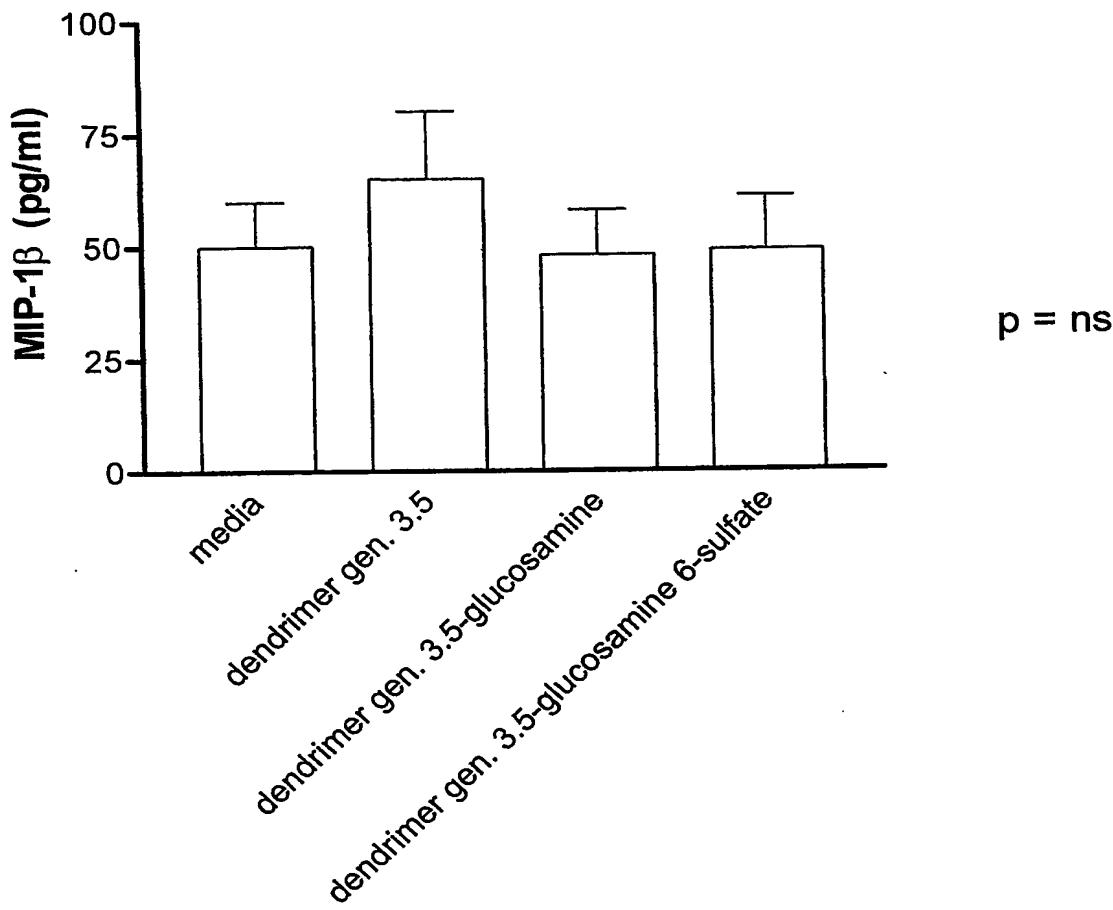
Figure 10(ii)



p = ns

- n = 3.
- single donor peritoneal macrophages.
- all compounds used at 25 μ g/ml.
- culture supernatants harvested at 36 h.

Figure 10(iii)



- n = 3.
- single donor peritoneal macrophages.
- all compounds used at 25 μ g/ml.
- culture supernatants harvested for MIP-1 β at 72 h.

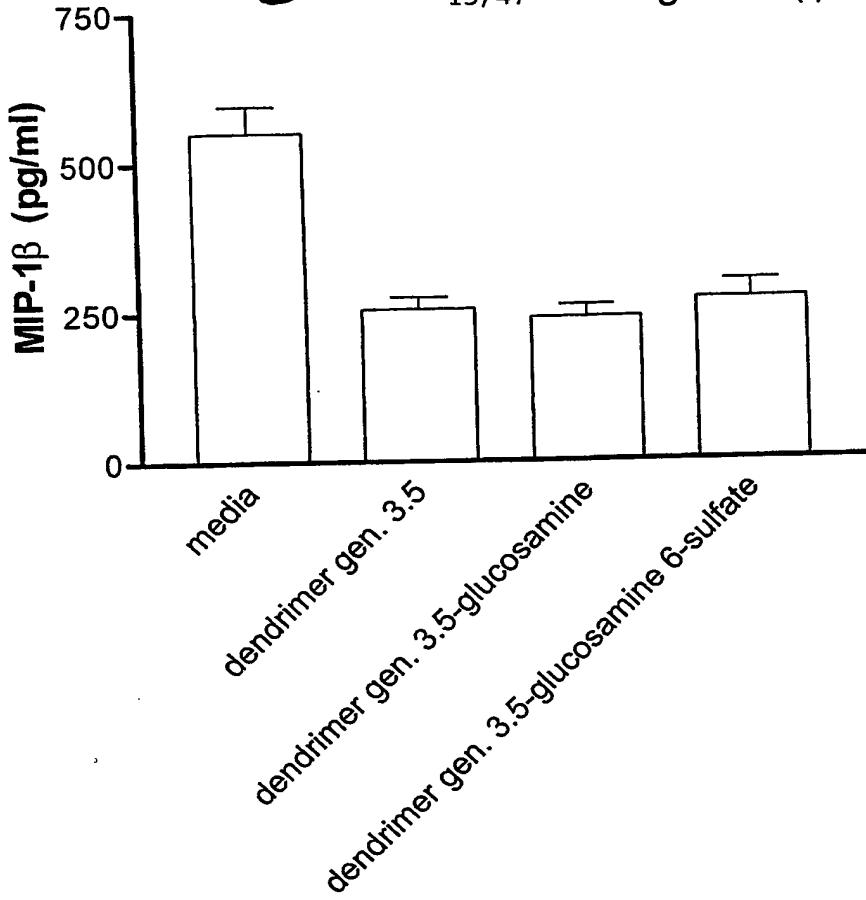


Figure 11(ii)

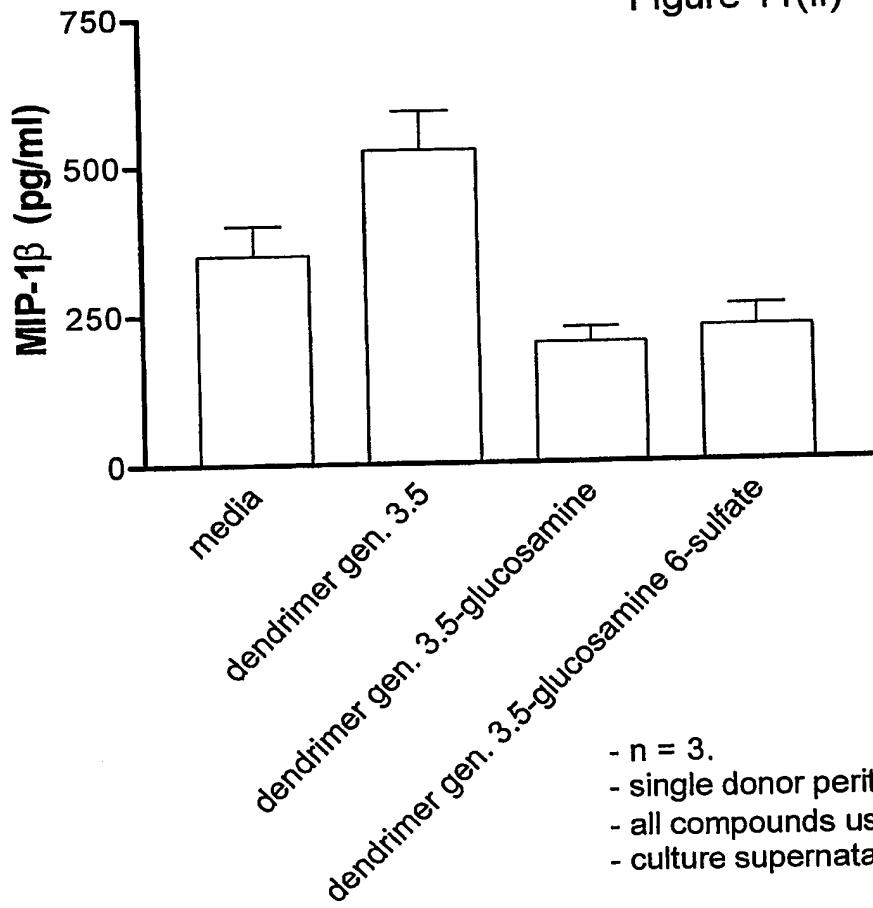


Figure 12 (i)

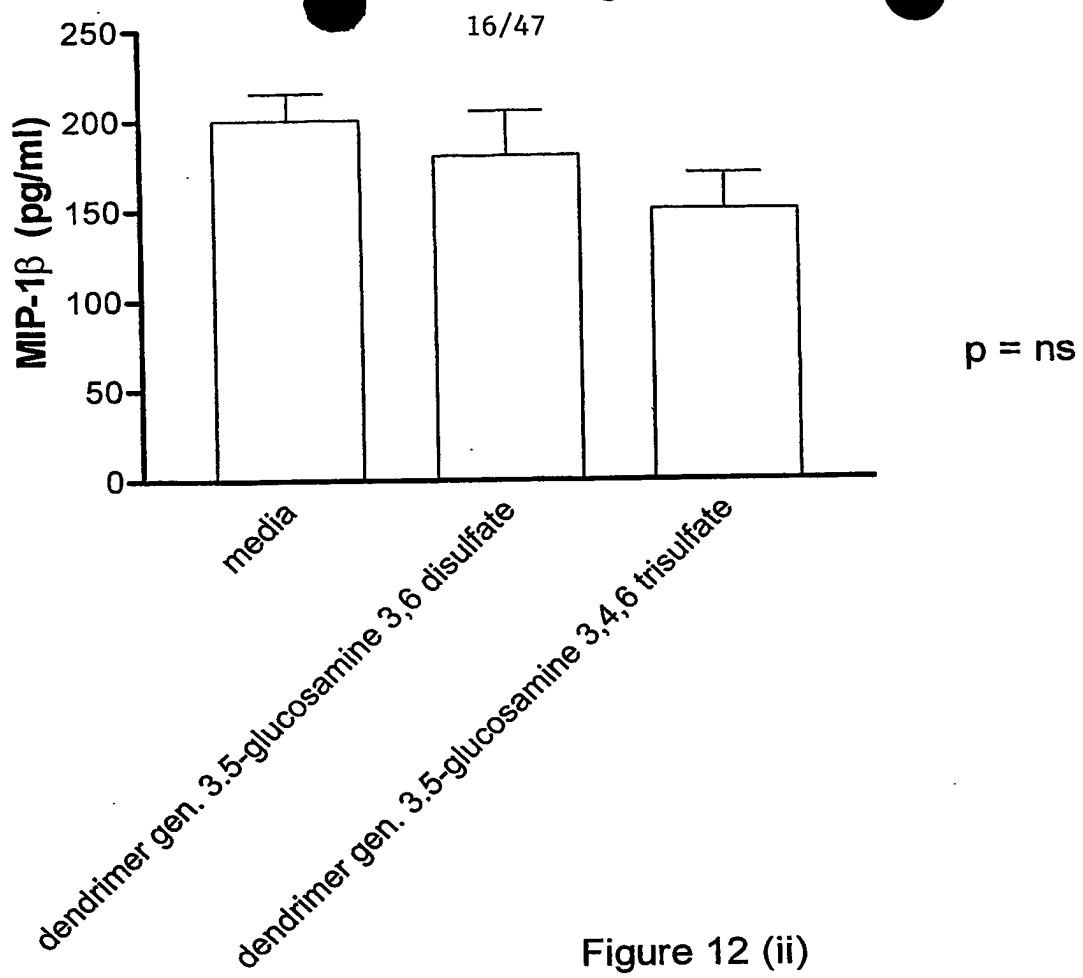


Figure 12 (ii)

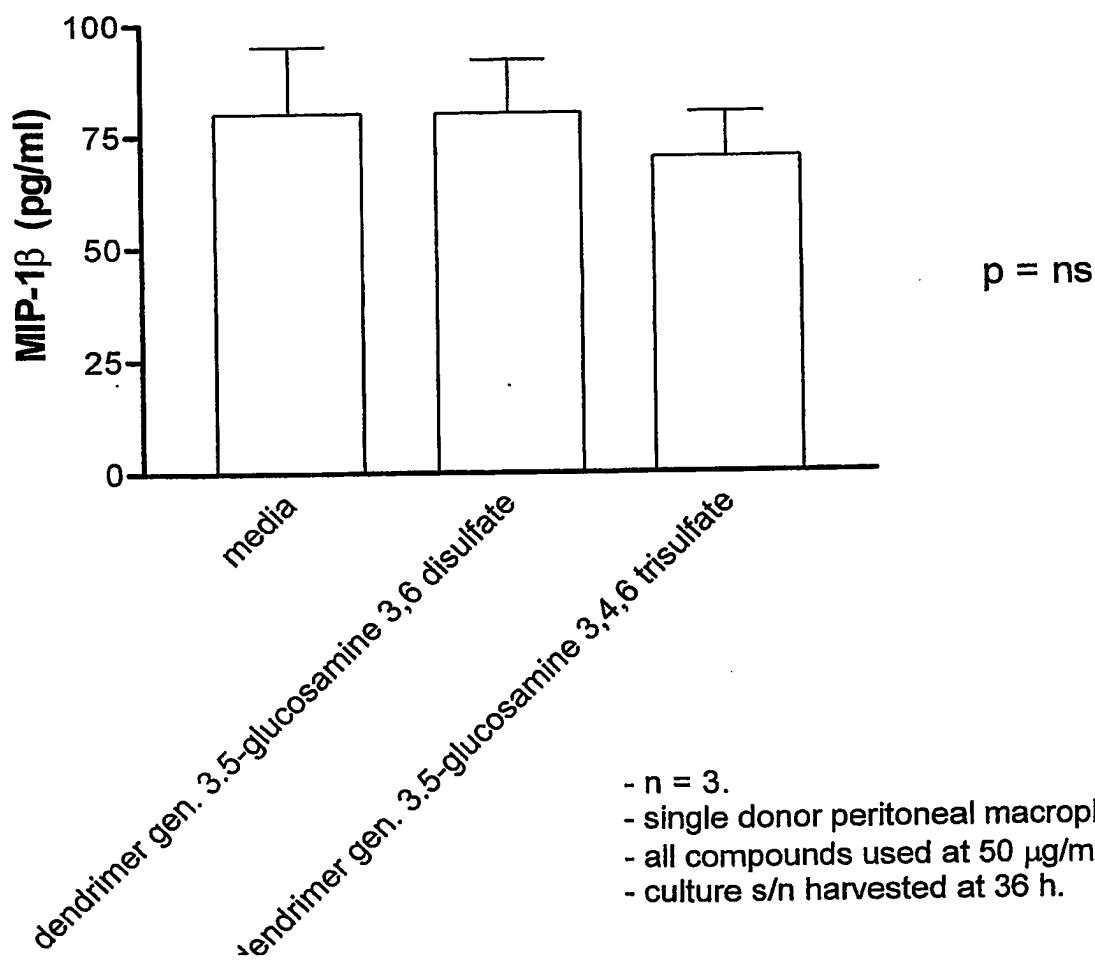


Figure 13 (i)

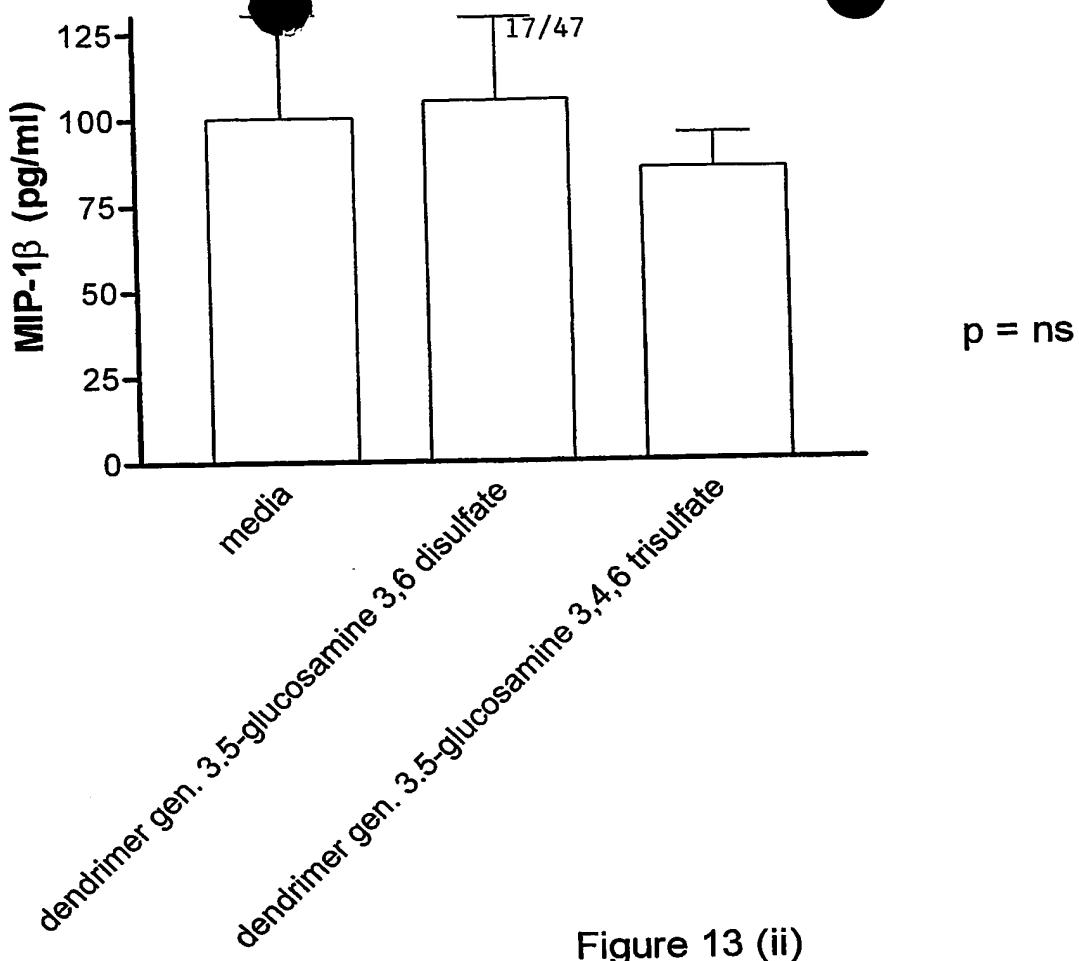
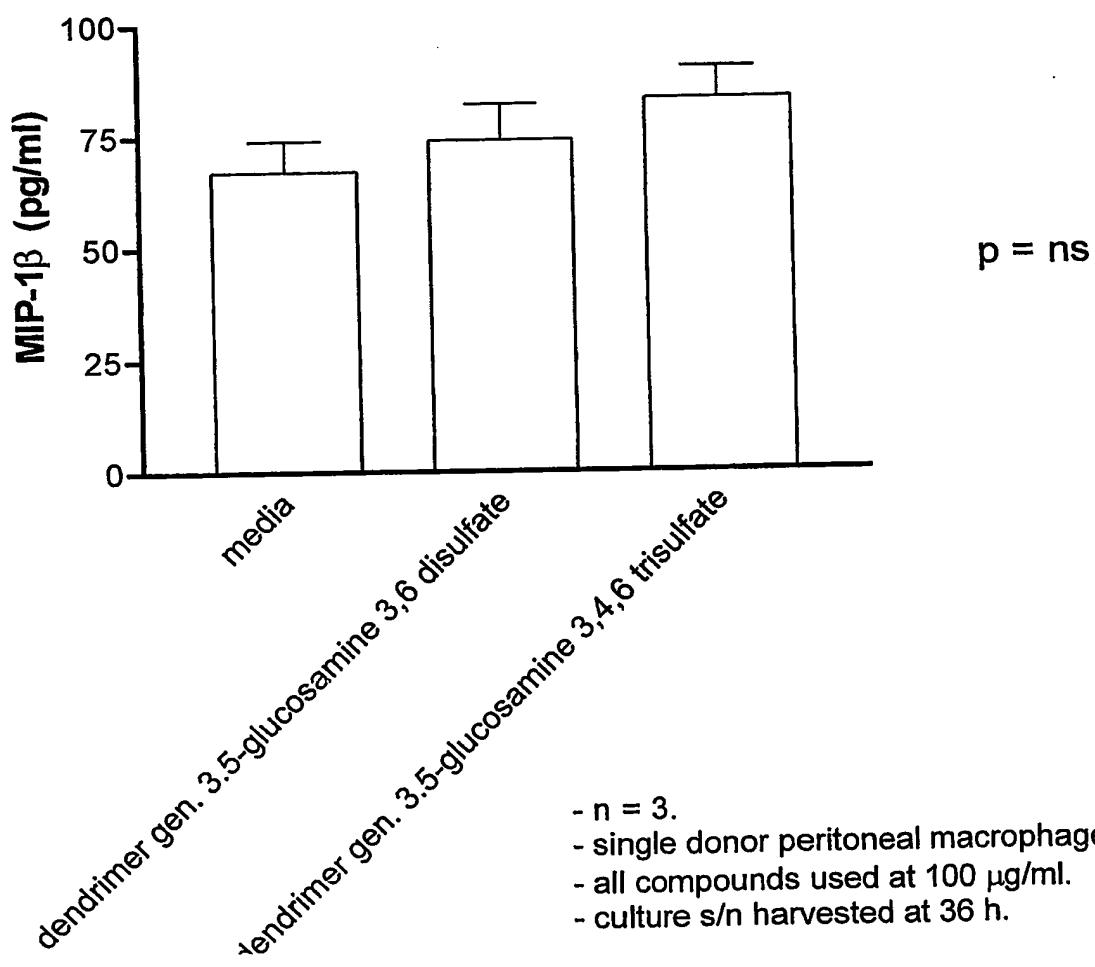


Figure 13 (ii)



- n = 3.
- single donor peritoneal macrophages.
- all compounds used at 100 μ g/ml.
- culture s/n harvested at 36 h.

Figure 13 (iii)

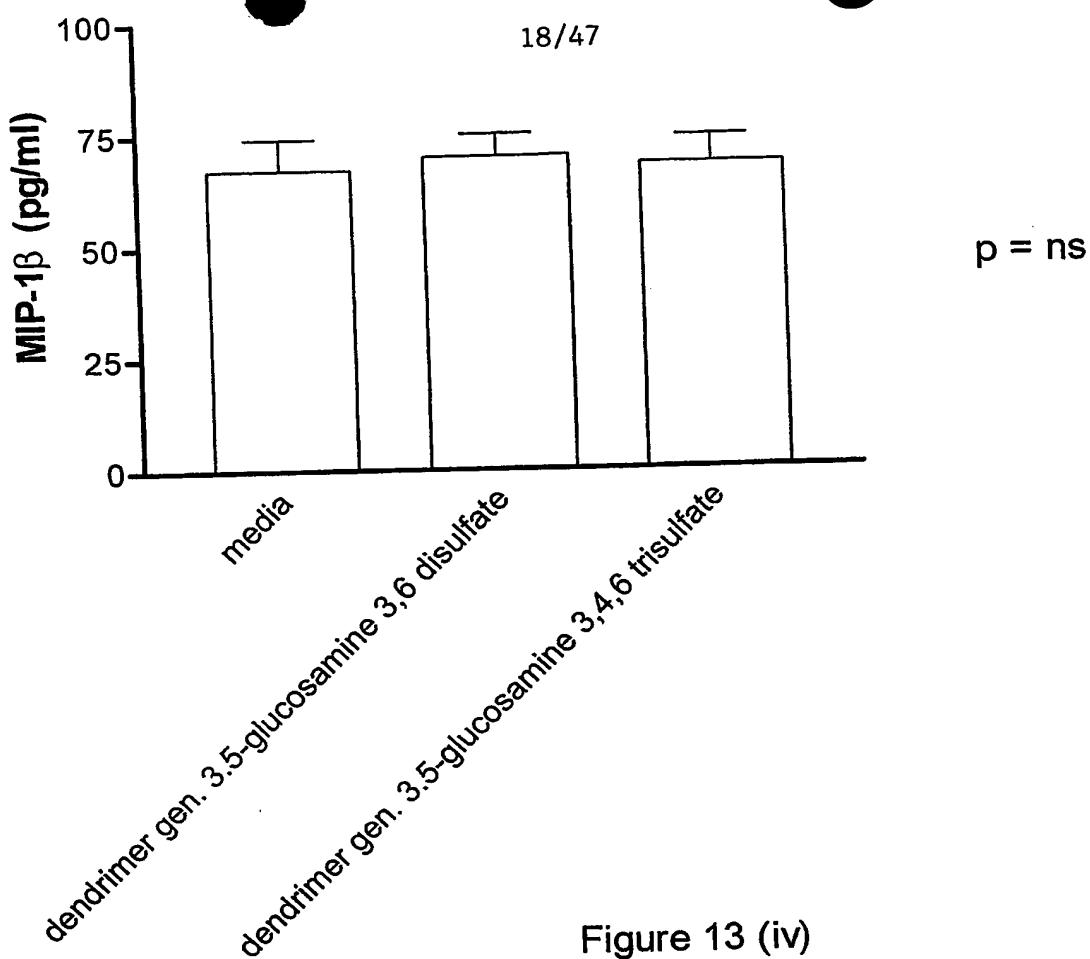


Figure 13 (iv)

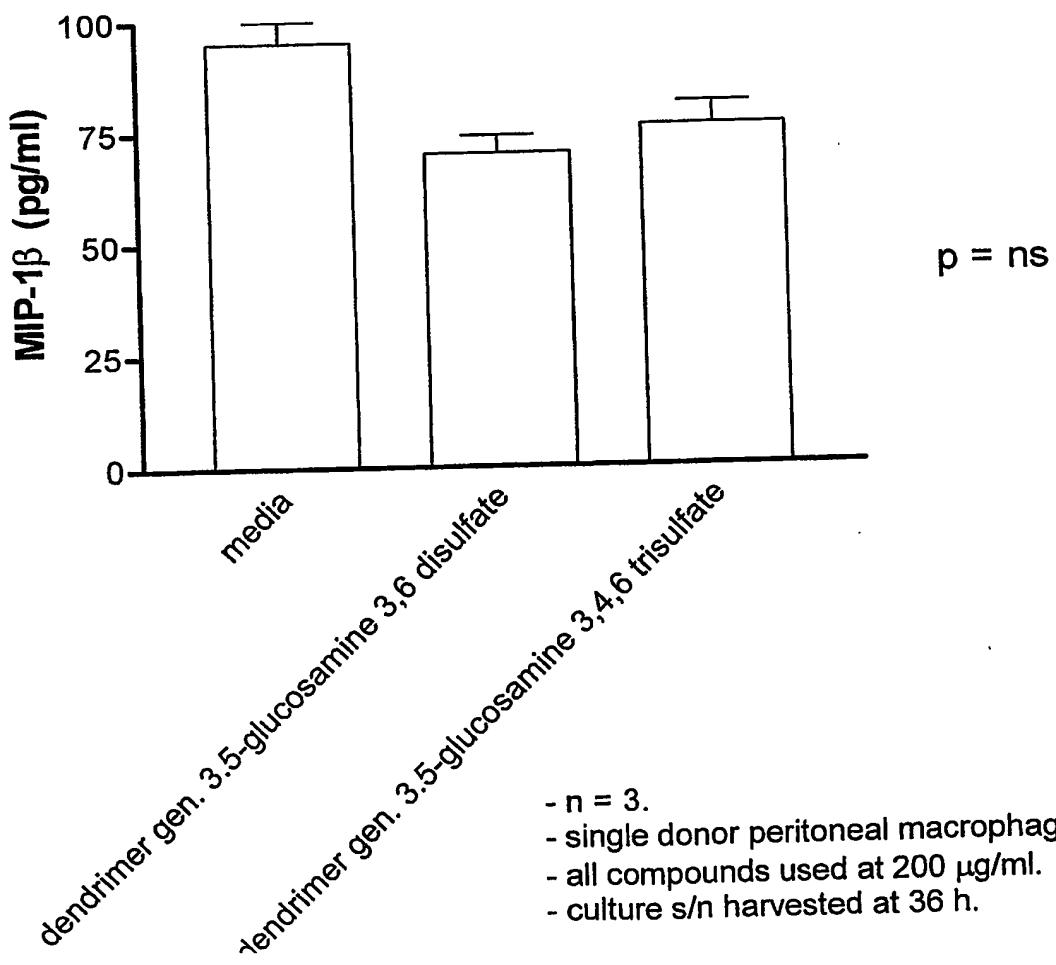
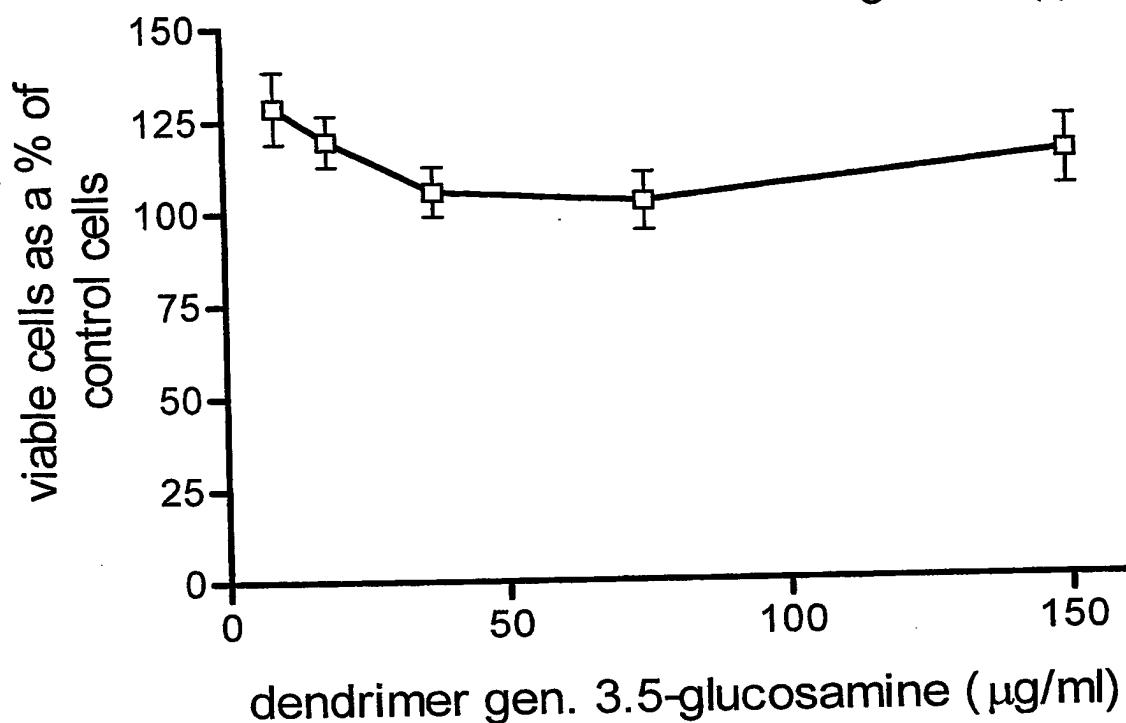
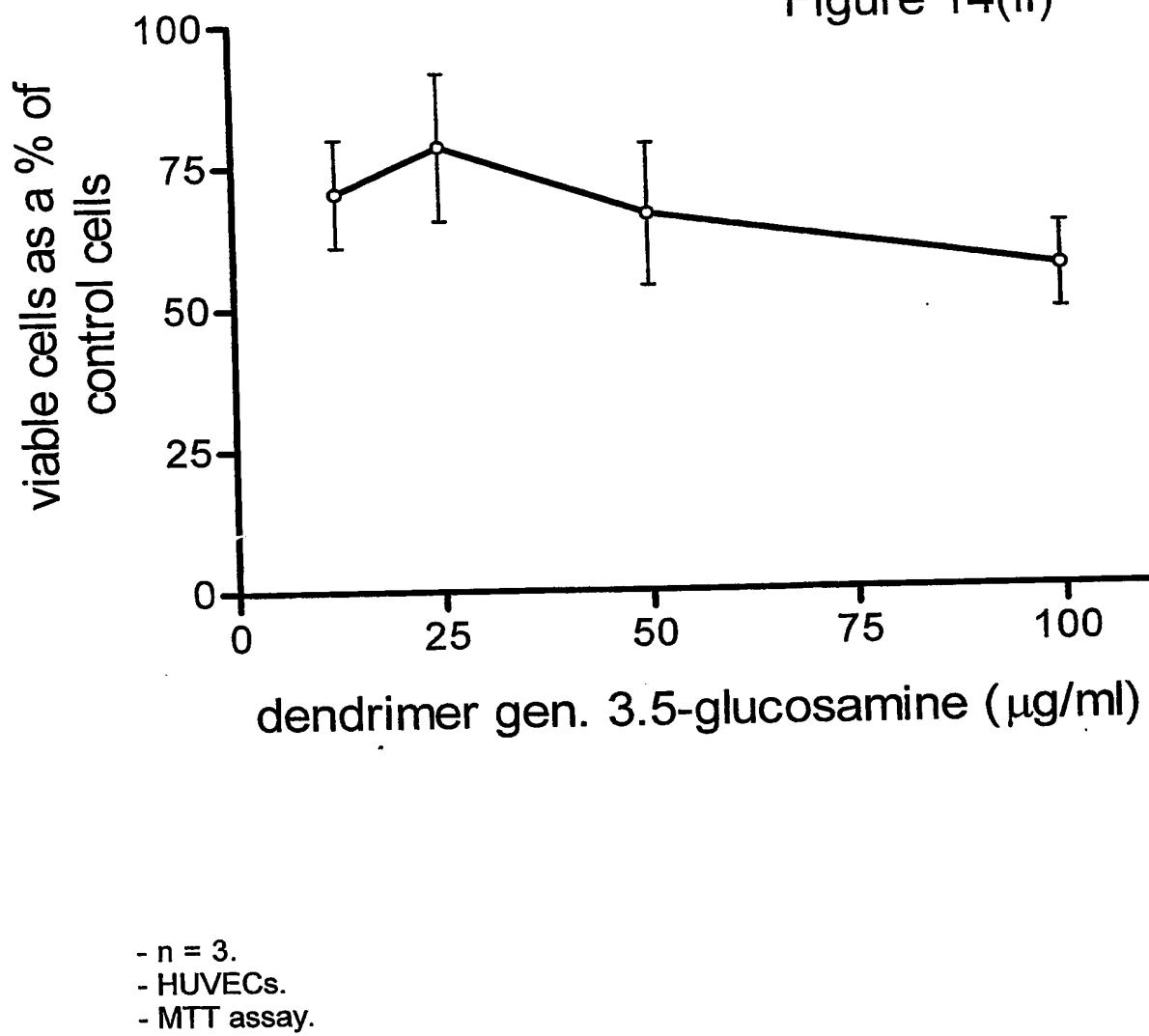


Figure 14(i)



- n = 3.
- single donor MDMs.
- MTT assay.

Figure 14(ii)



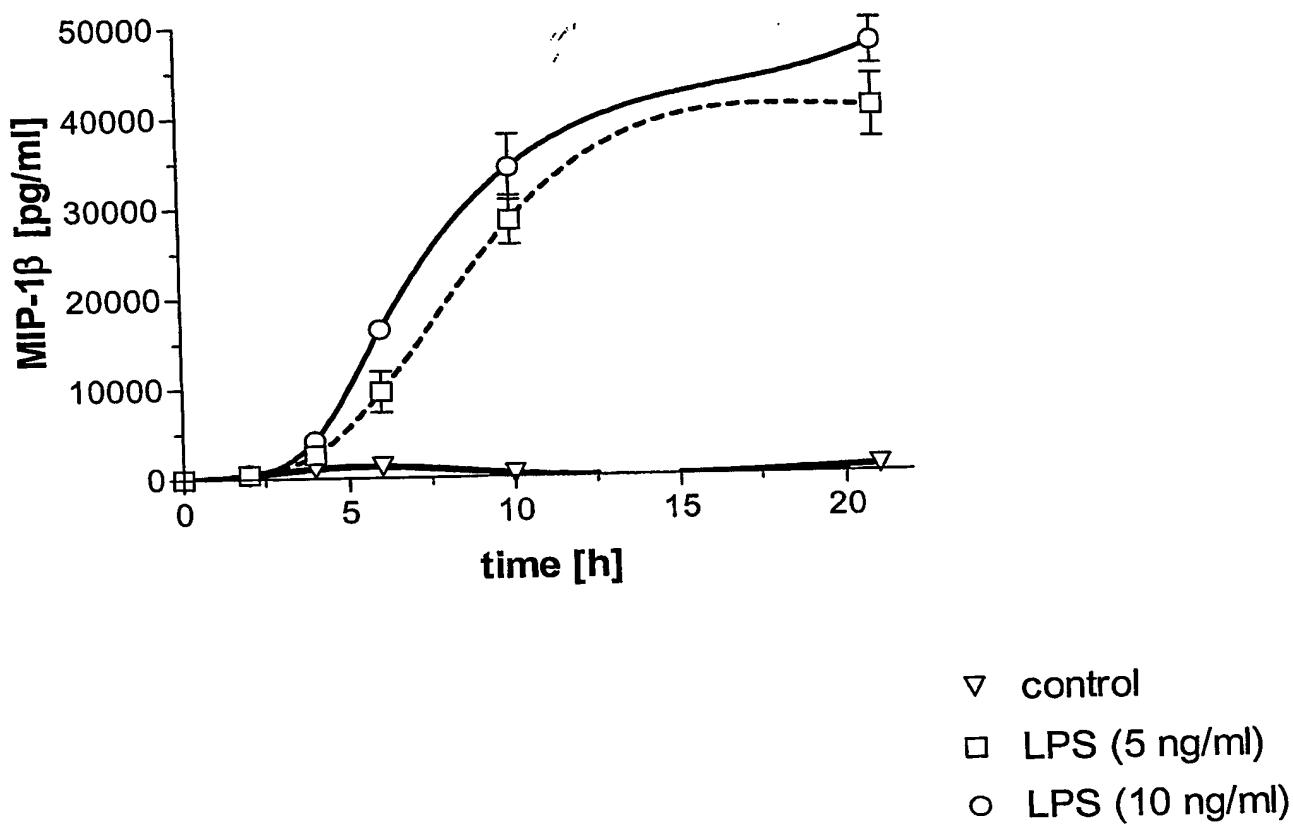


Figure 15:
Time course of the release of MIP-1 β into cell free culture supernatants
after the addition of LPS at a final concentration of 5 ng/ml & 10 ng/ml.

Figure 16(i)
LPS added 30 min after dendrimer gen 3.5-glucosamine

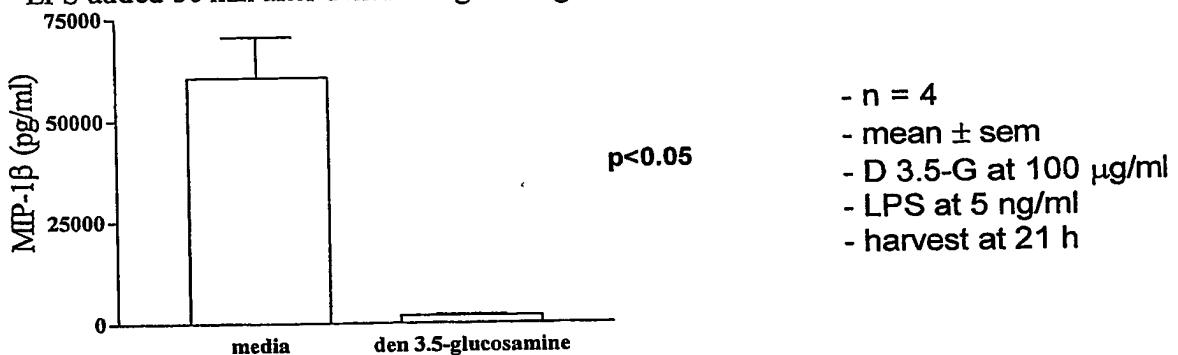
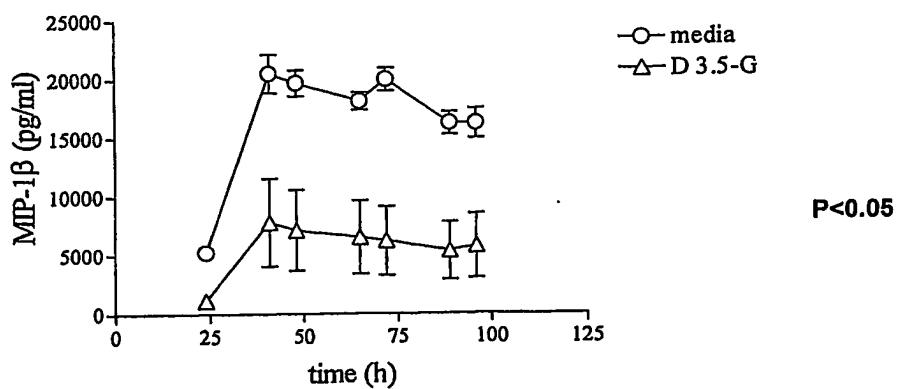


Figure 16(ii)
LPS added 1 h after
dendrimer gen 3.5-glucosamine



Figure 16(iii)
LPS was added 24 h after dendrimer gen 3.5-glucosamine



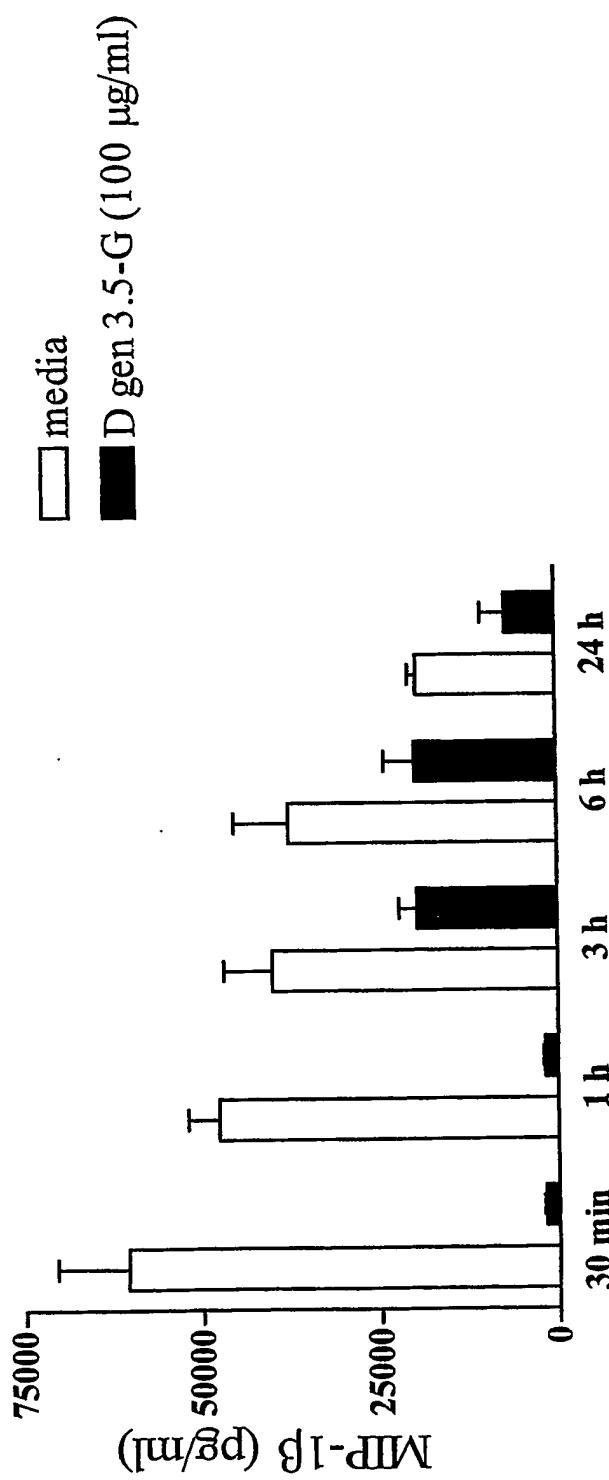


Figure 16(iv)

- Release of MIP-1 β from single donor PBMN cells. n = 4.
- LPS (5 ng/ml) was added at various time points ranging from 30 min to 24 h after dendrimer gen. 3.5-glucosamine (100 μ g/ml).
- Culture supernatants harvested 21 h after the addition of LPS for MIP-1 β .

(D gen 3.5-G: dendrimer gen 3.5-glucosamine)

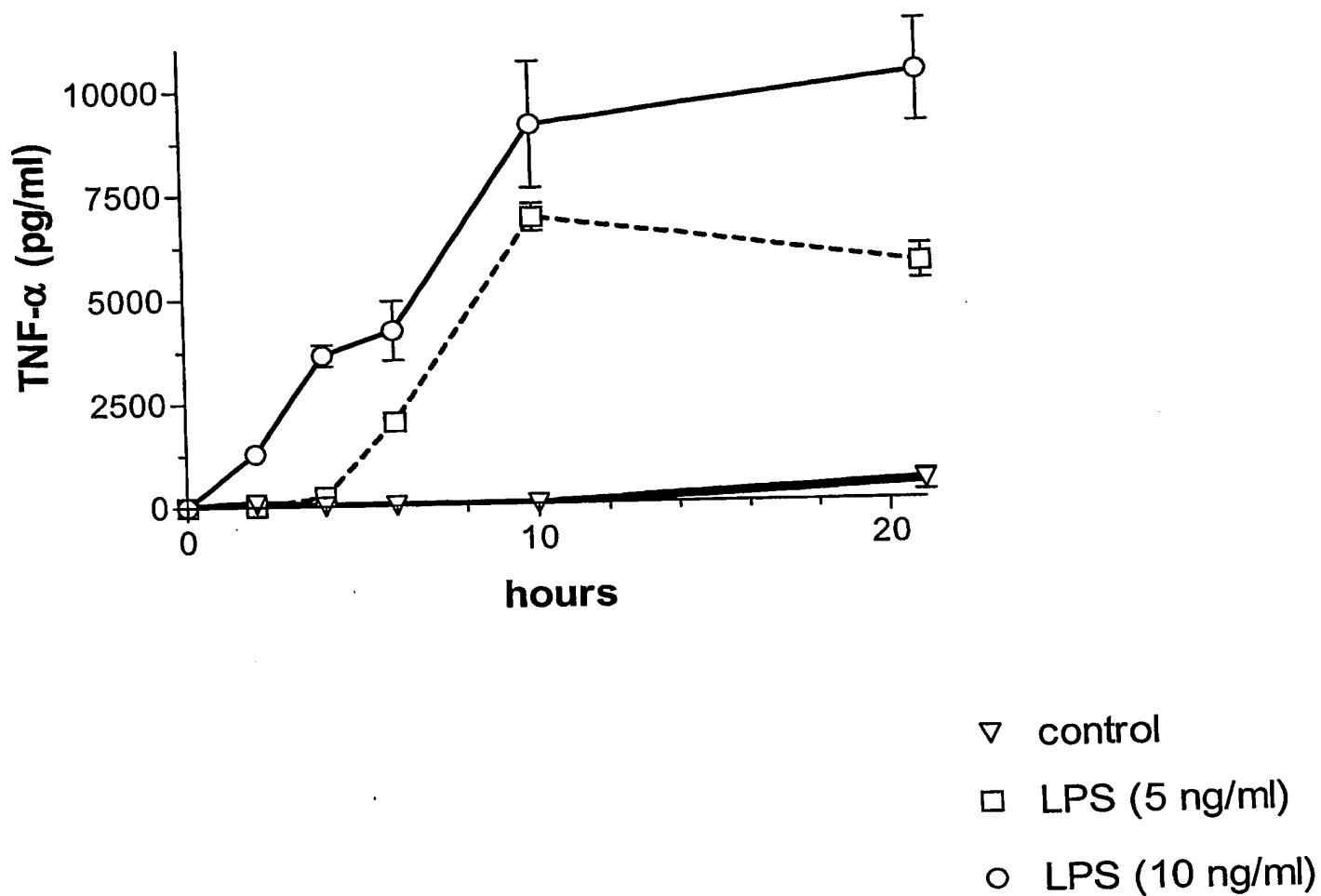


Figure 17:

Time course of the release of TNF- α into cell free culture supernatants following the addition of LPS at a final concentration of 5 ng/ml and at 10 ng/ml.

Figure 18:
LPS (5 ng/ml) was added 30 min after dendrimer gen 3.5-glucosamine (100 μ g/ml) and culture supernatants harvested at 21 h.
(P<0.05 for all 6 figures)

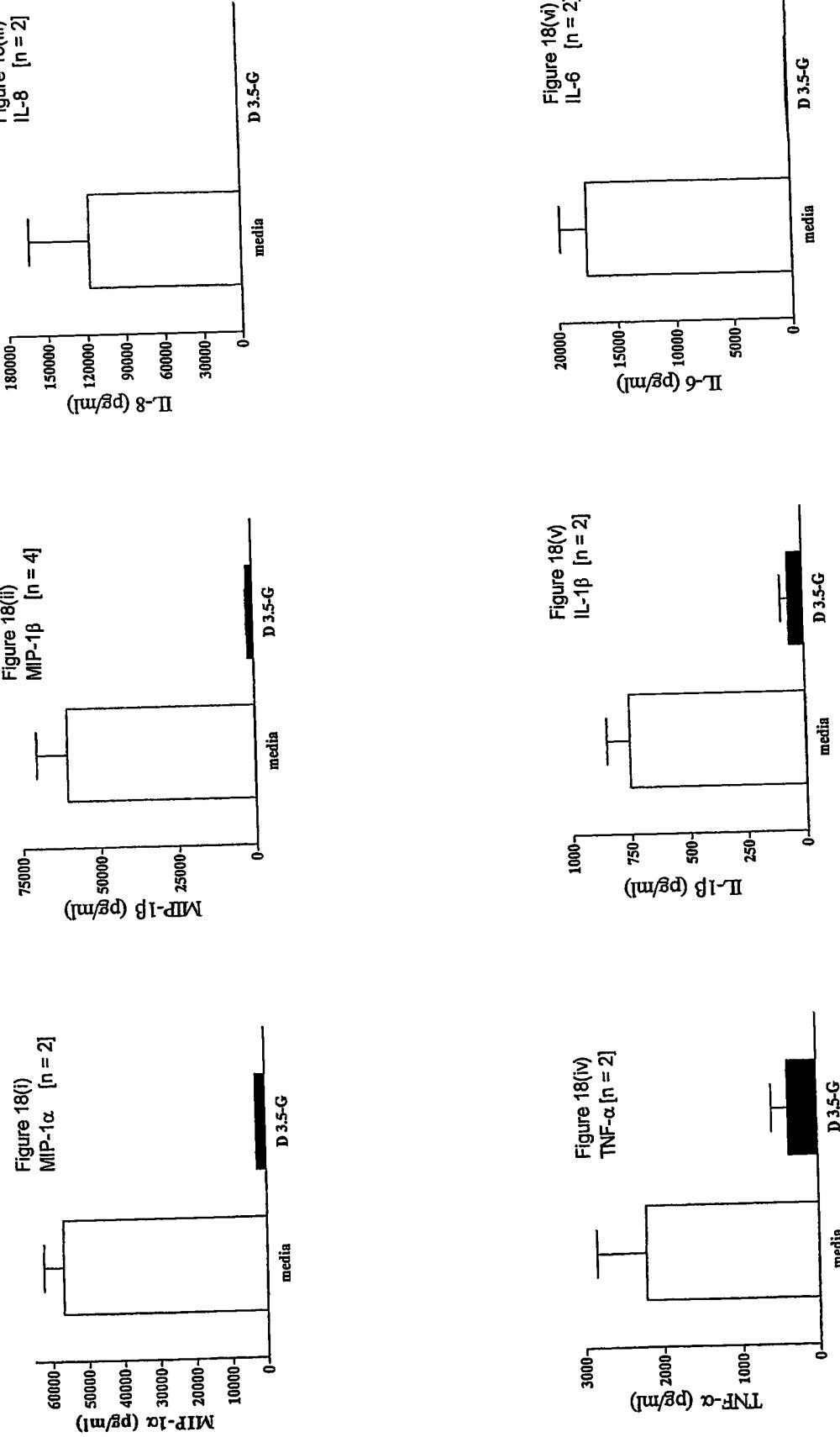


Figure 19:
LPS (5 ng/ml) was added 30 min after dendrimer gen 3.5-glucosamine (200 μ g/ml) and culture supernatants harvested at 25 h.
($P < 0.05$ for all groups)

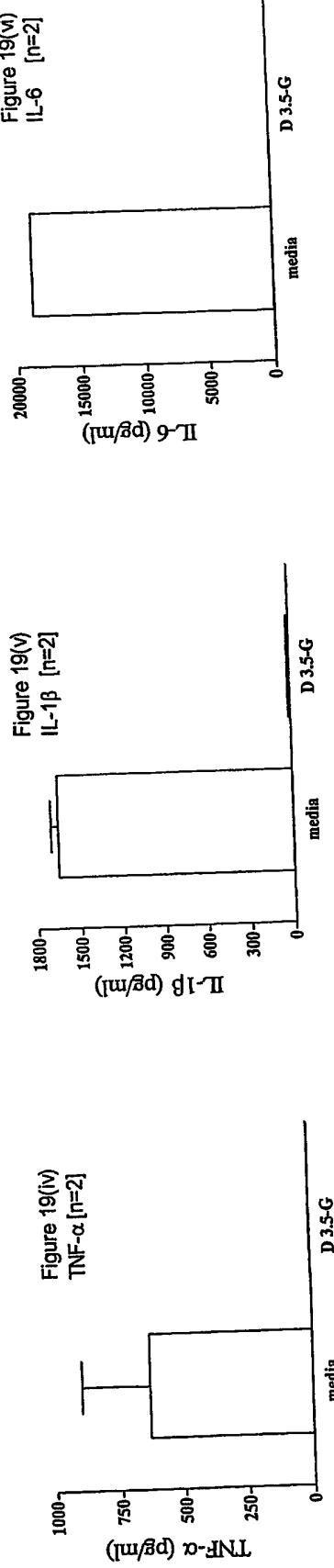
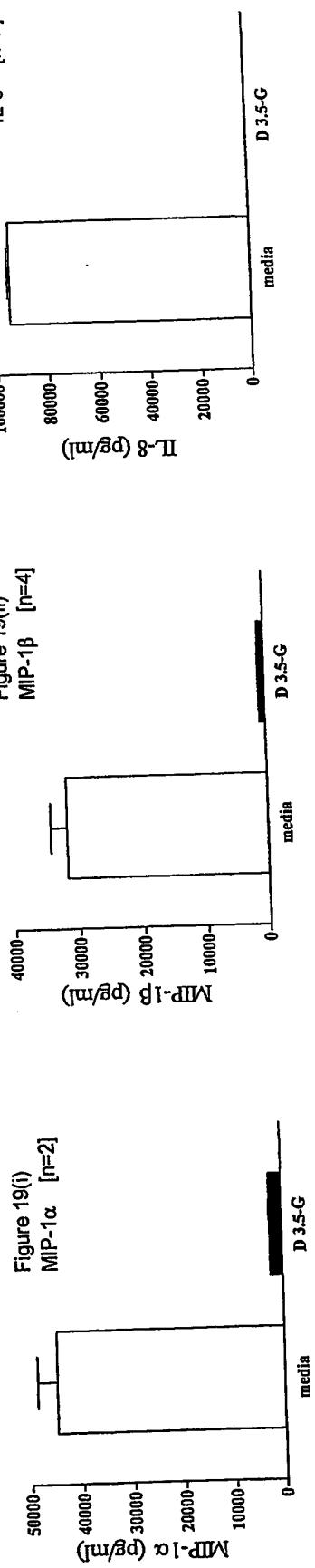
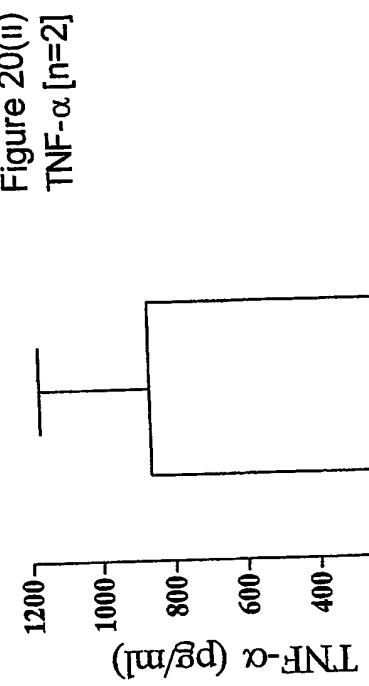
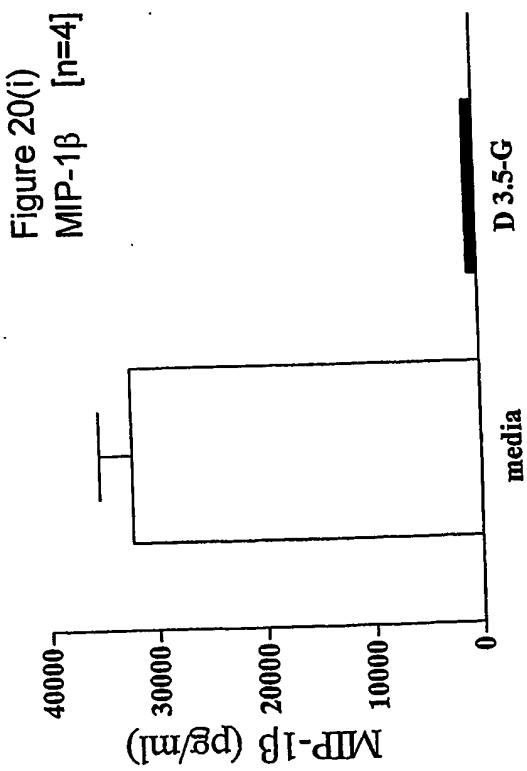


Figure 20:
LPS (5 ng/ml) added 1 hour after dendrimer gen 3.5-glucosamine (200 μ g/ml) and culture supernatants harvested at 25 h.
(P<0.05 for all groups)



mean \pm sem

Figure 21(i): Dendrimer (D 3.5-G) 3.5-glucosamine ((D 3.5 G at 100 μ g) added 30 min or 1 h or 2 h or 3 h or 4 h after LPS (5 ng/ml). Culture supernatants harvested at 21 h.

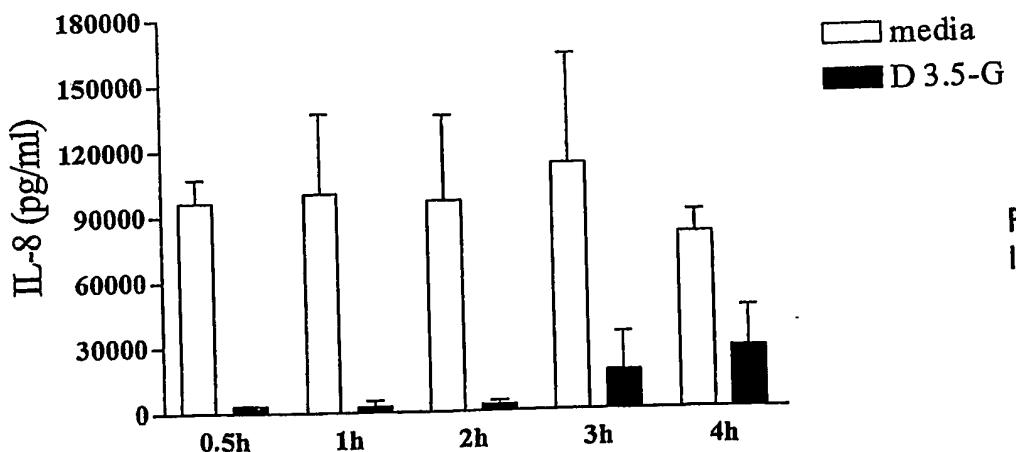
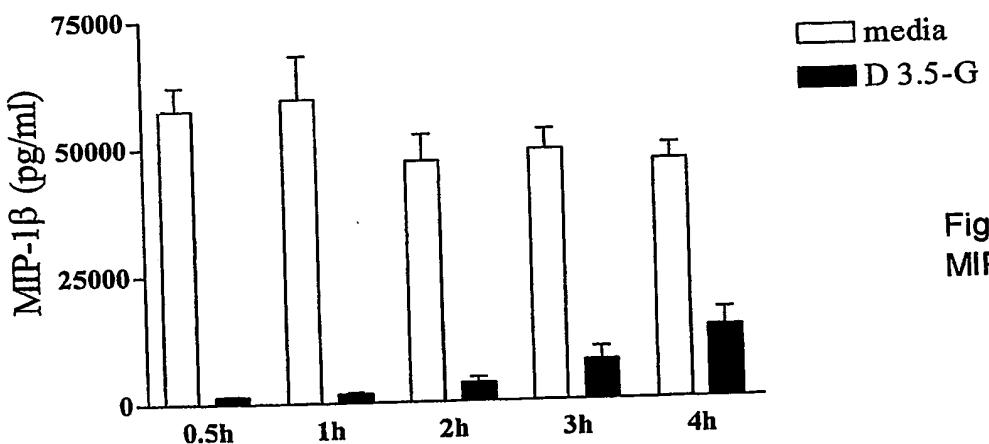
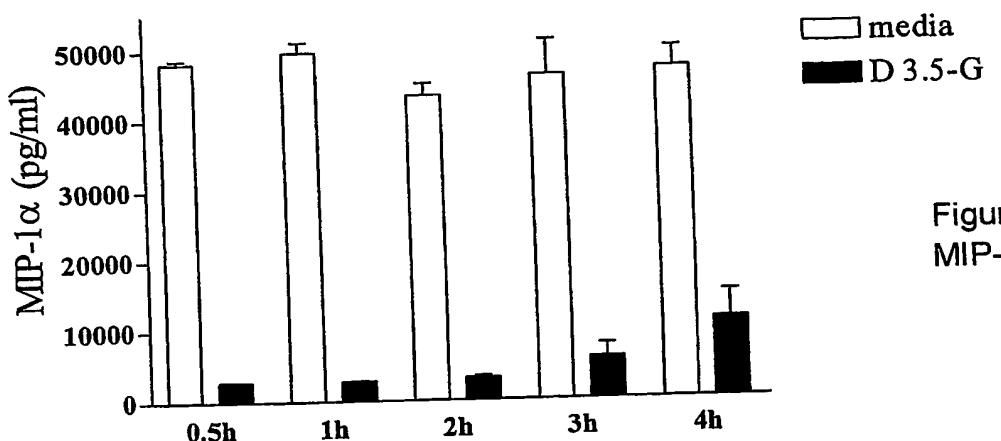


Figure 21(ii): Dendrimer gen. 3.5-glucosamine (100 μ g/ml) added 30 min or 1 h or 2 h or 3 h or 4 h after LPS (5 ng/ml). Culture supernatants harvested at 21 h.

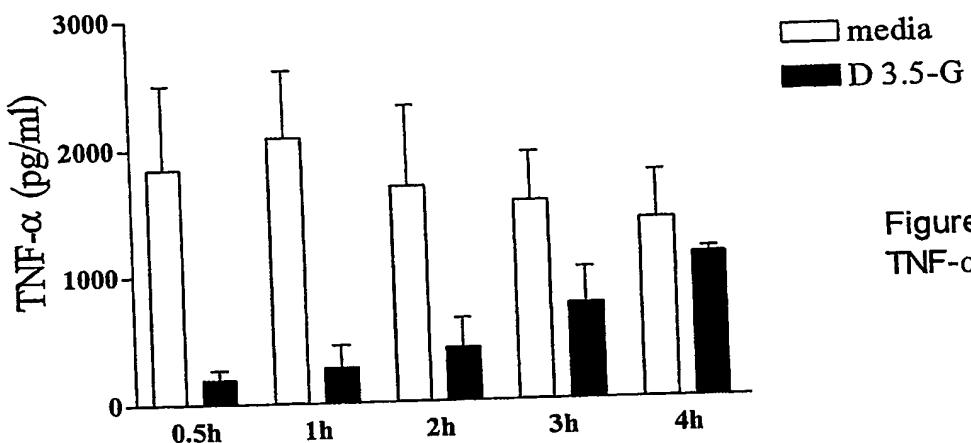


Figure 21(iv)
TNF- α [n=2]

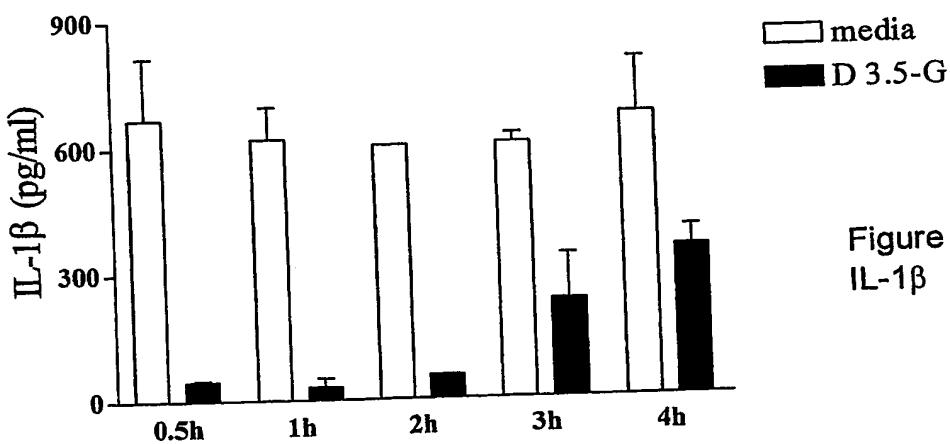


Figure 21(v)
IL-1 β [n=2]

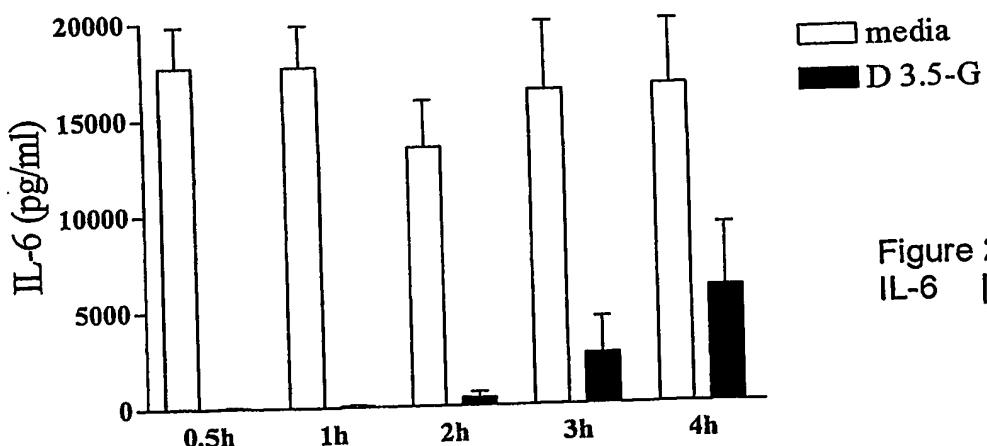


Figure 21(vi)
IL-6 [n=2]

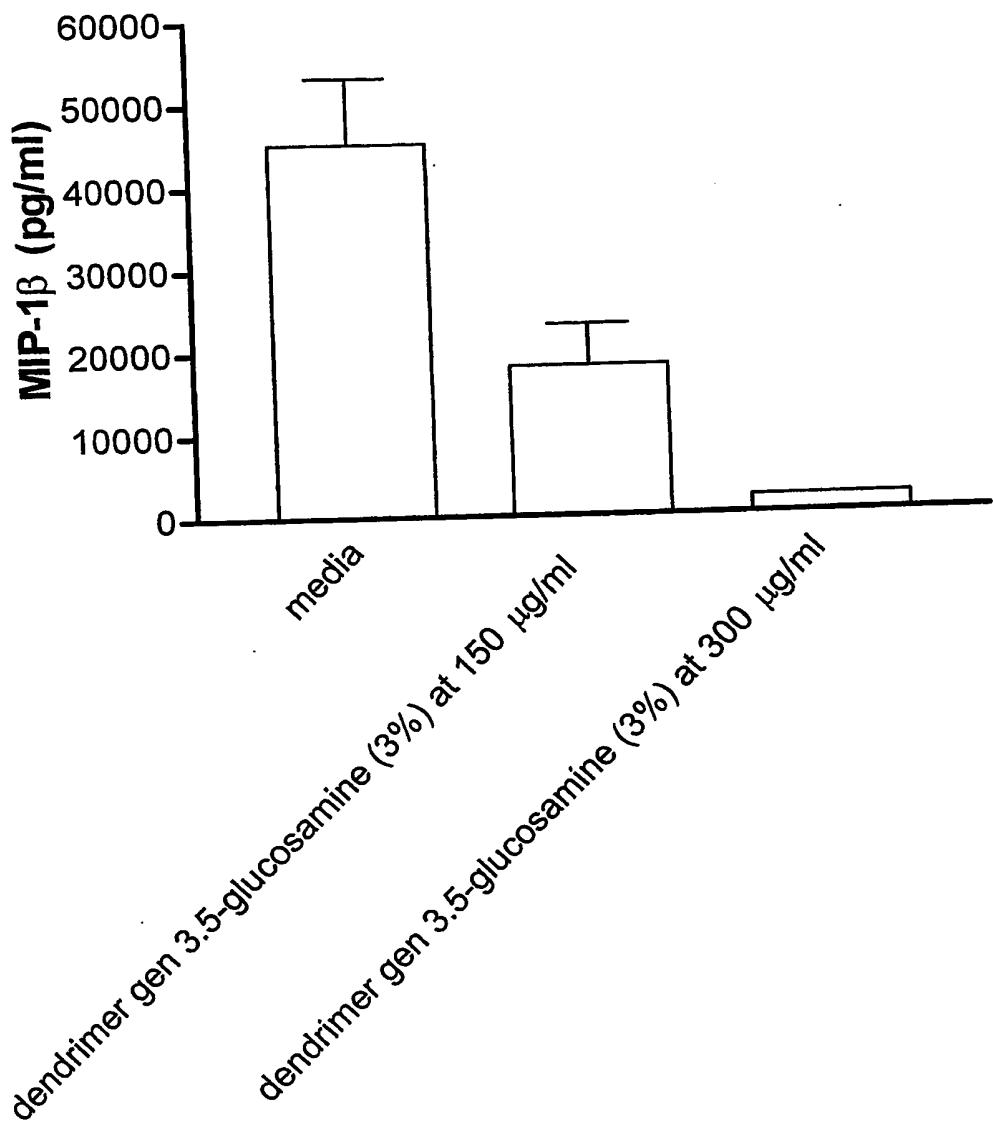


Figure 22(i):
Release of MIP-1 β from single donor PBMN cells. LPS (5ng/ml) was added 30 min after the dendrimer generation 3.5-glucosamine (3% loading) at a concentration of either 150 μ g/ml or 300 μ g/ml. Culture supernatants were harvested at 22 h (n=1).

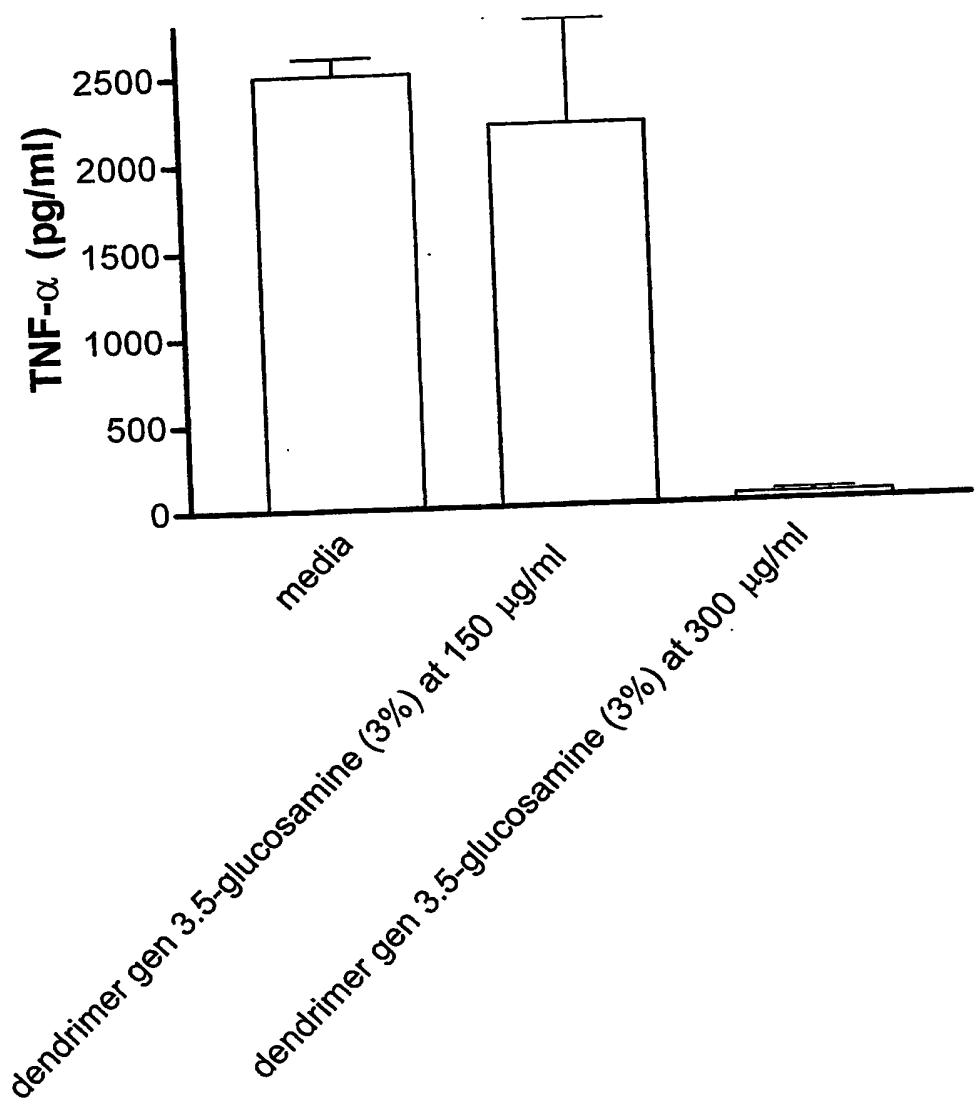


Figure 22(ii):
Release of TNF- α from single donor PBMN cells. LPS (5ng/ml) was added 30 min after the dendrimer generation 3.5-glucosamine (3% loading) at a concentration of either 150 μ g/ml or 300 μ g/ml. Culture supernatants were harvested at 22 h (n=1).

Figure 23(i)
PBMN cells from 3 donors mixed [n=2]

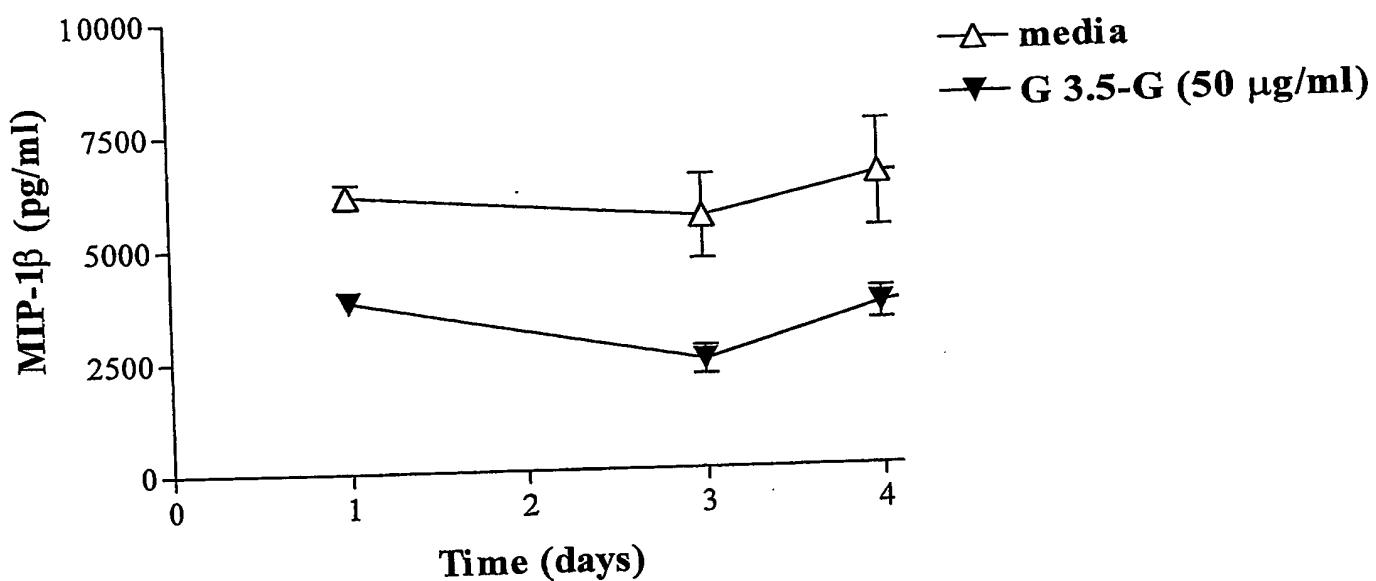


Figure 23(ii)
PBMN cells from 3 donors mixed [n=2]

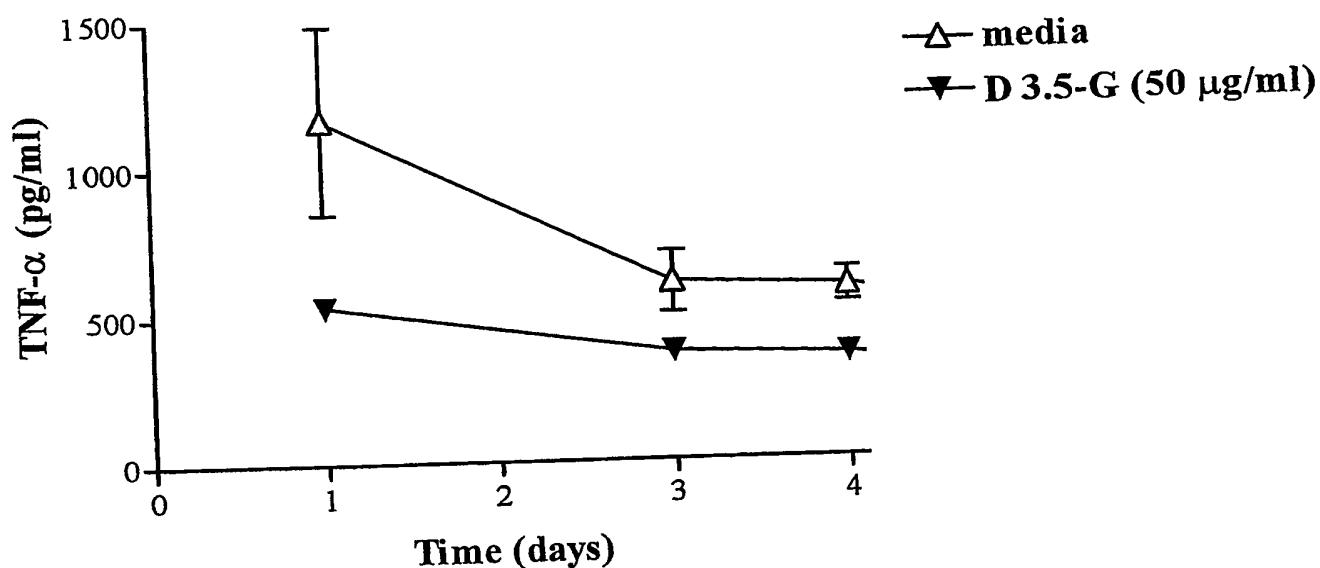


Figure 23(iii)
PBMN cells from 2 donors mixed [n=2]

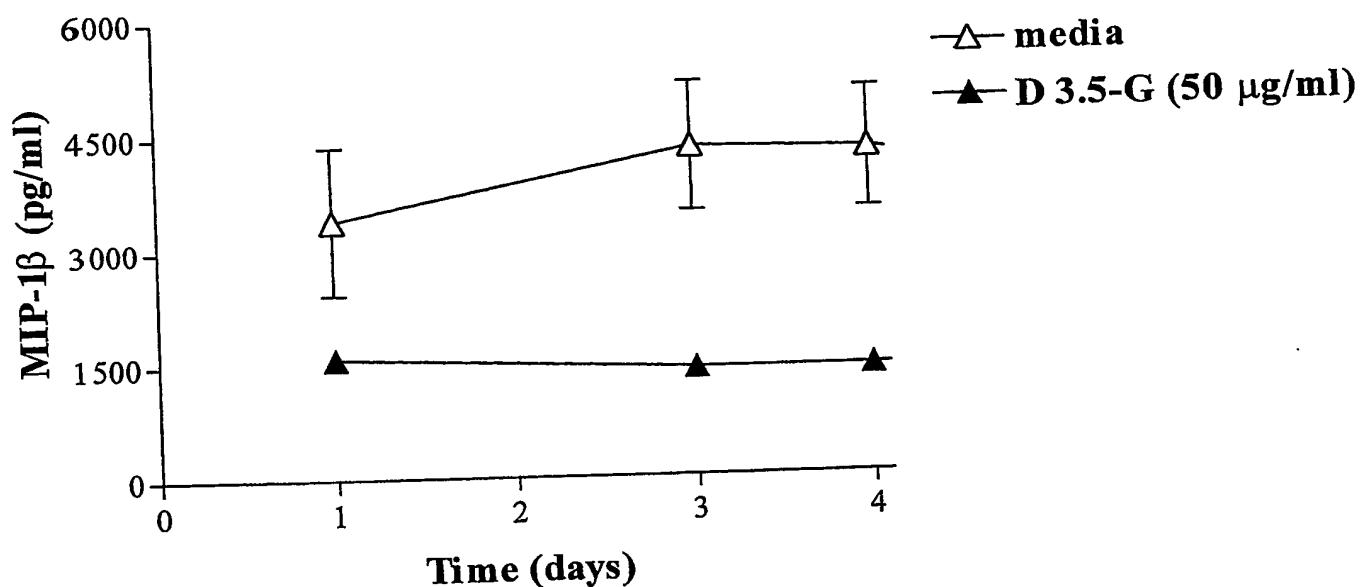
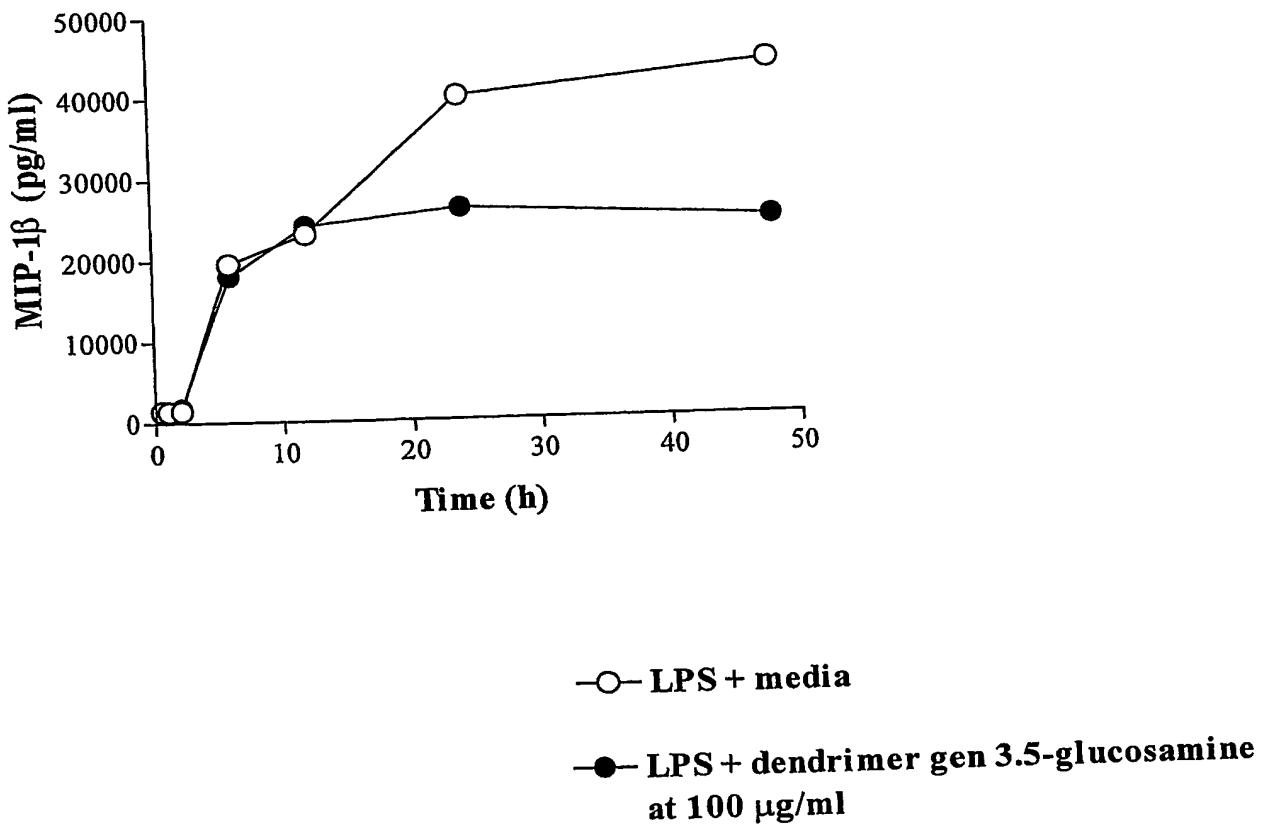
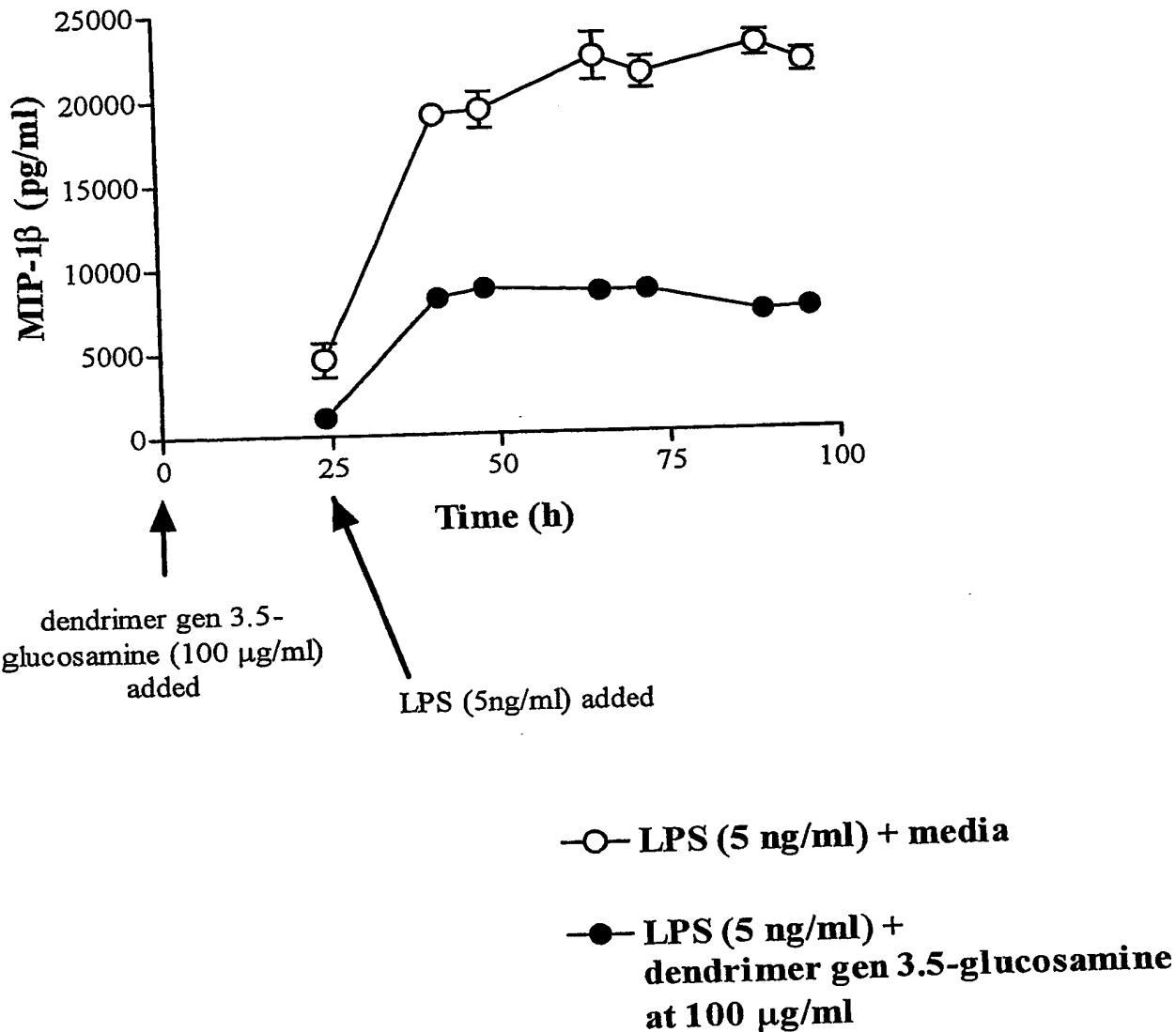


Figure 24(i)
PBMN cells from 2 donors mixed [n=2]



Donor PBMN cells from 2 individuals mixed,
Dendrimer gen 3.5-glucosamine (100 μ g/ml)
followed immediately by LPS at 10 ng/ml).

Figure 24(ii) 36/47



PBMC cells from 2 individuals were mixed and the dendrimer gen 3.5-glucosamine added at 100 μ g/ml. 24 hours later, LPS was added at 5 ng/ml (n=2).

Figure 25(i)
PBMN cell viability when cultured with compounds for up to 5 days.

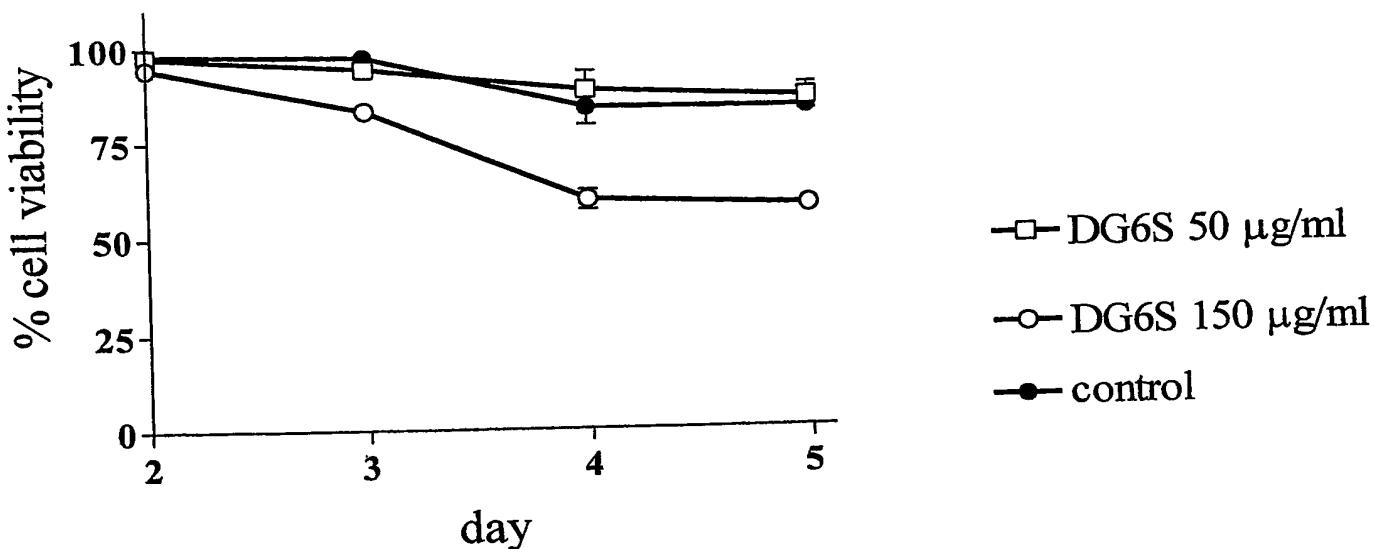


Figure 25(ii)
PBMN cell counts when cultured with compounds for up to 5 days.

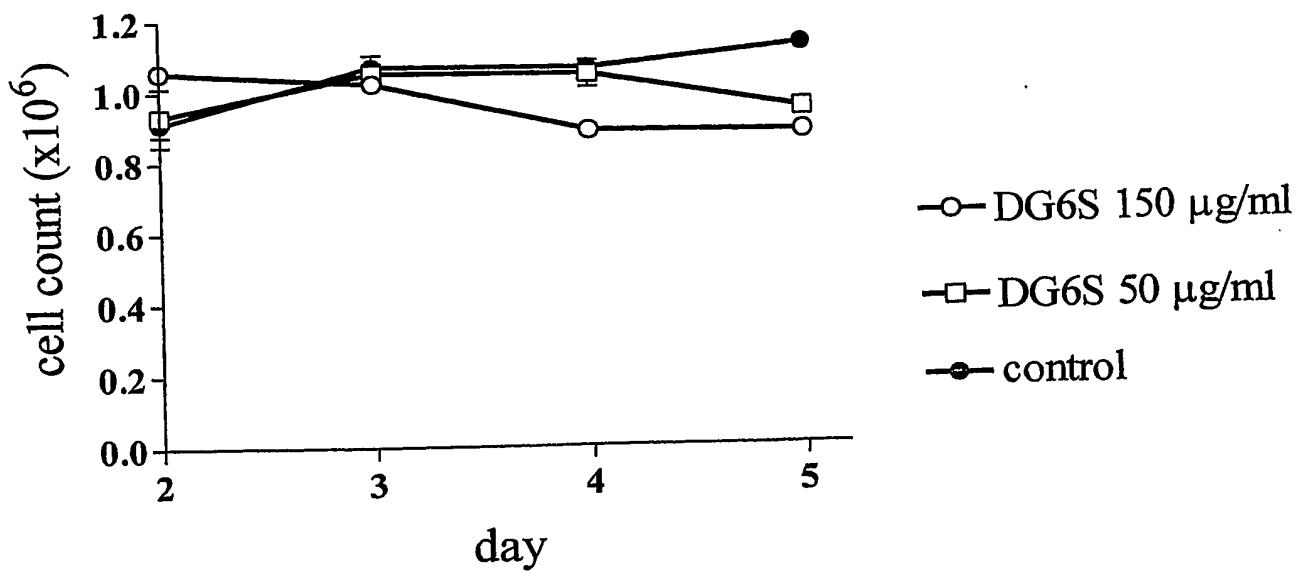


Figure 26:
Cell viability of MDMs when cultured with
dendrimer gen 3.5-glucosamine 6-sulfate over a period of 5 days

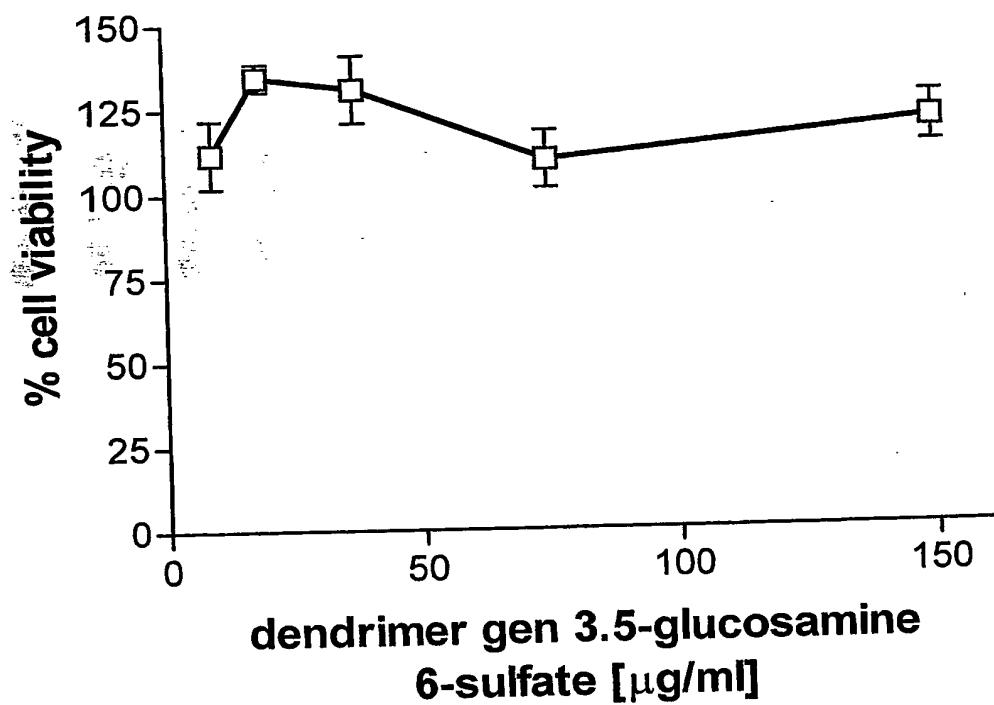


Figure 27:

mean \pm sem (n=8)

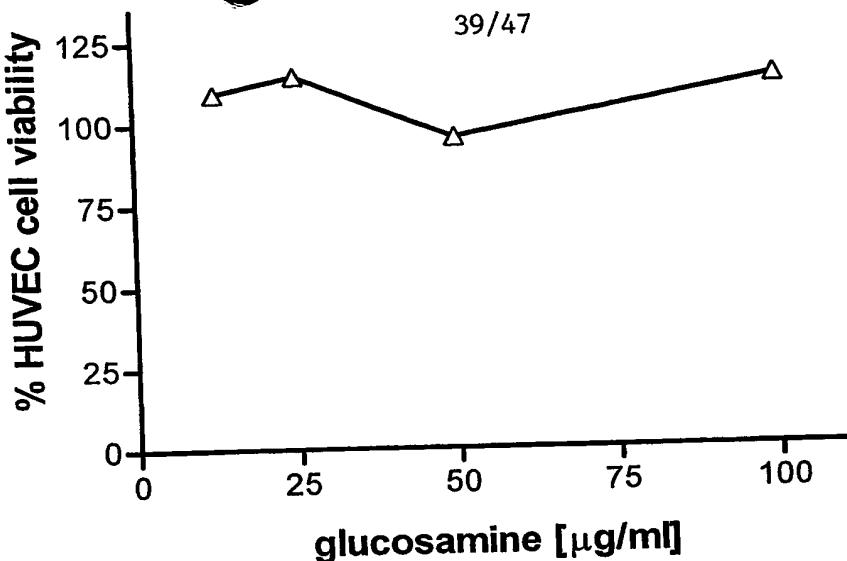


Figure 27 (i)

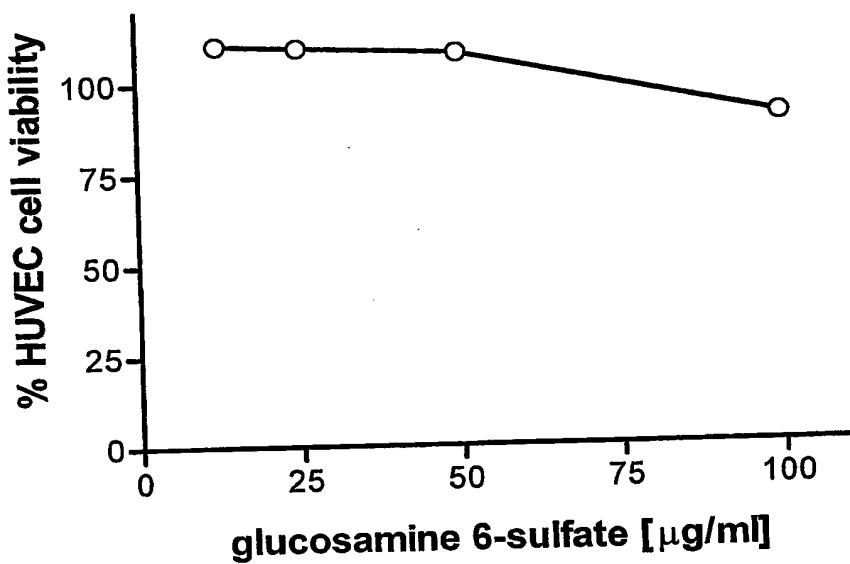


Figure 27 (ii)

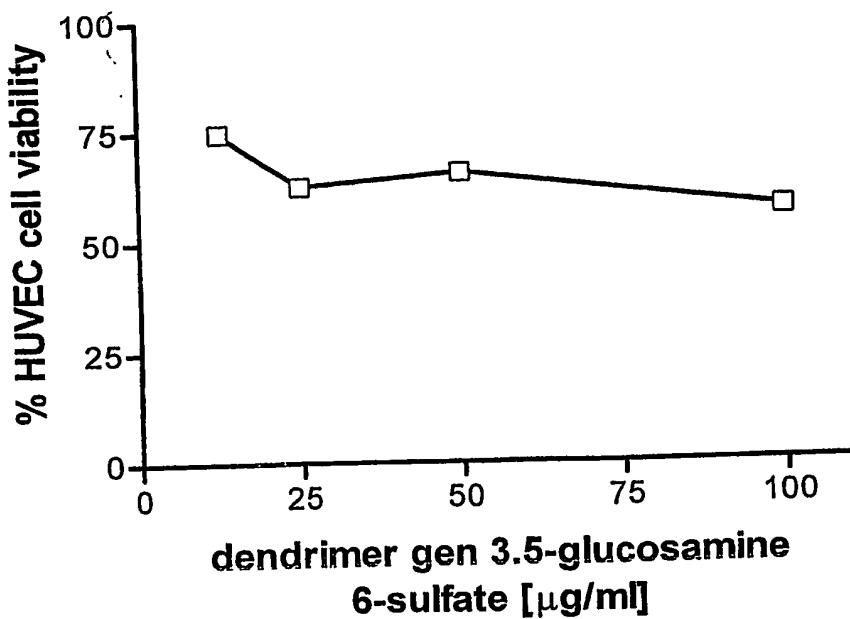


Figure 27 (iii)

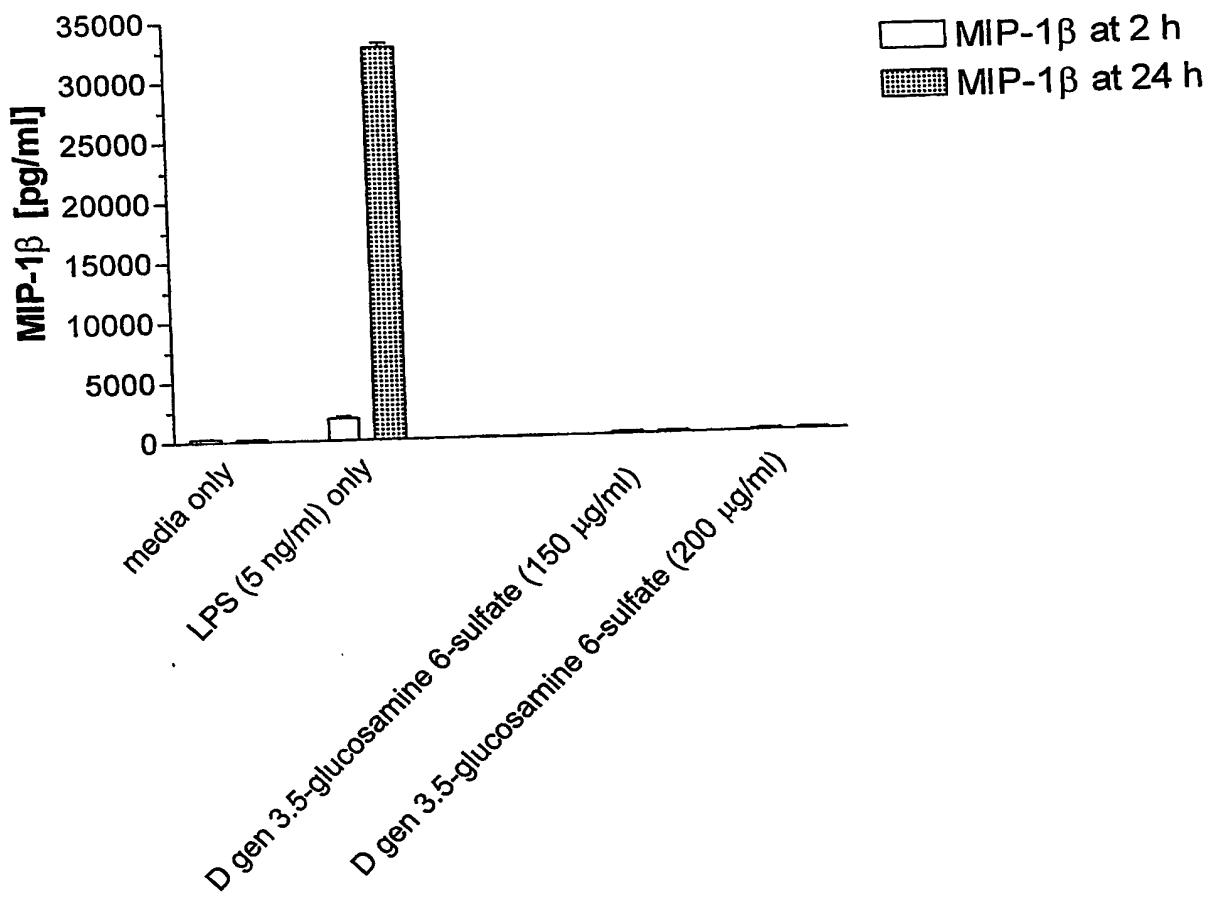


Figure 28(i):
MIP-1 β release from single donor PBMN cells when treated with
dendrimer gen 3.5-glucosamine 6-sulfate at 150 μ g/ml or 200 μ g/ml for 30 min
before the addition of LPS at 5 ng/ml.

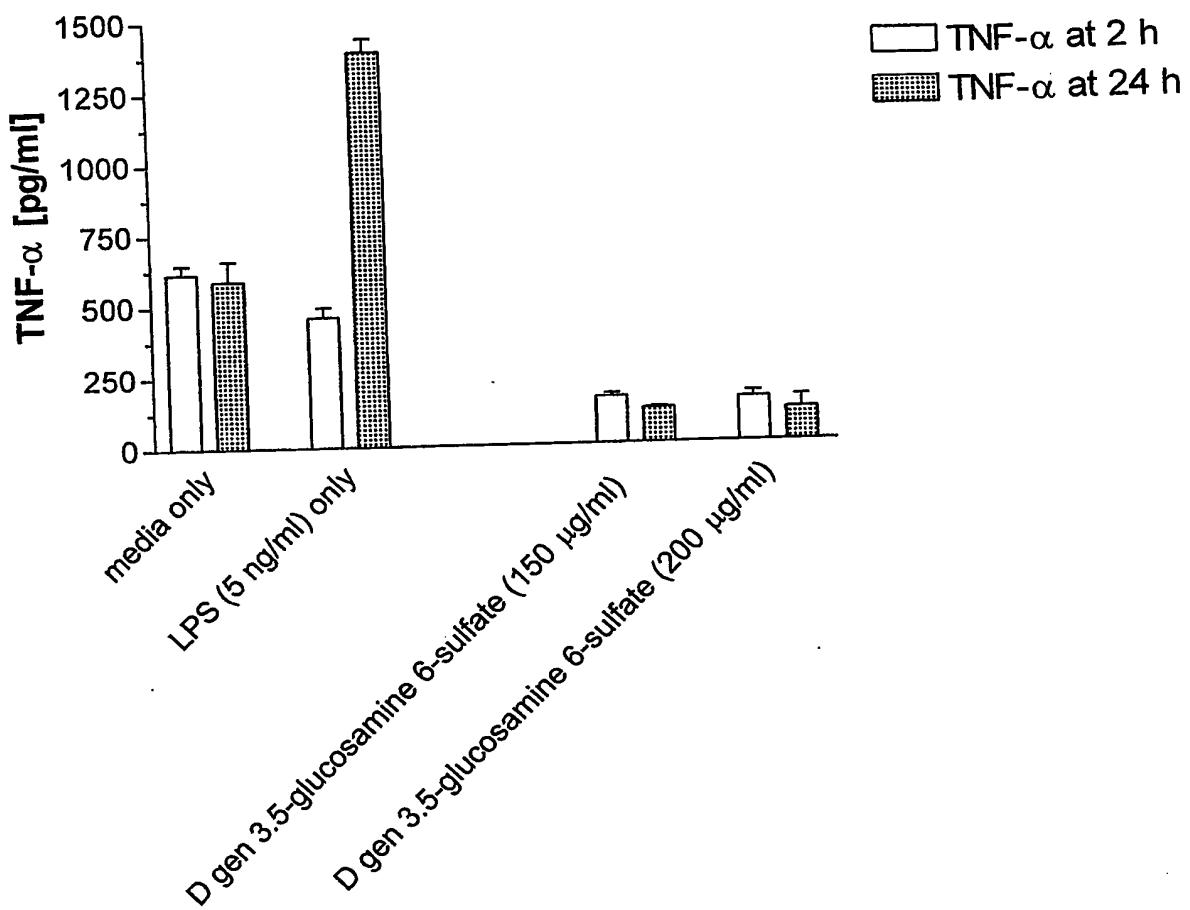


Figure 28(ii):
TNF- α release from single donor PBMN cells when treated with
dendrimer gen 3.5–glucosamine 6-sulfate at 150 μ g/ml or 200 μ g/ml for 30 min
before the addition of LPS at 5 ng/ml.

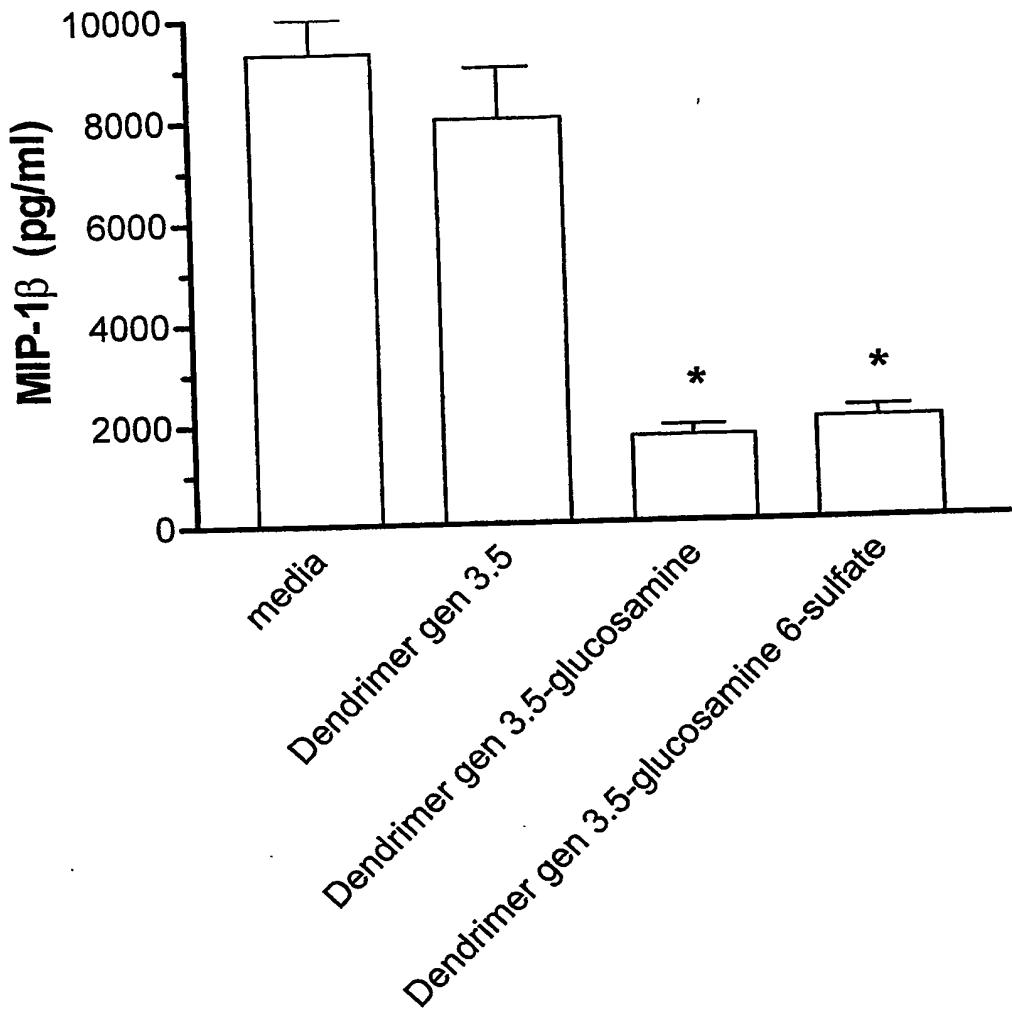


Figure 29:
MDMs from four donors were pooled for 24 h and each of the compounds shown then added at a concentration of 125 μ g/ml. Cell free culture supernatants were then harvested at 36 h for measurement of MIP-1 β . A reduction in MIP-1 β was seen when dendrimer gen 3.5 glucosamine or dendrimer gen 3.5-glucosamine 6-sulfate was present.

* $p < 0.05$ compared to the media control

Figure 30: Endothelial microtubule formation by HUVECs on Matrigel (x 40 magnification). A visual analogue scale was used to determine the extent of tube formation and scored on a scale from 0 (all cells remain single) to 4 (all cells involved in tubular structures) as described in the text.

Control well

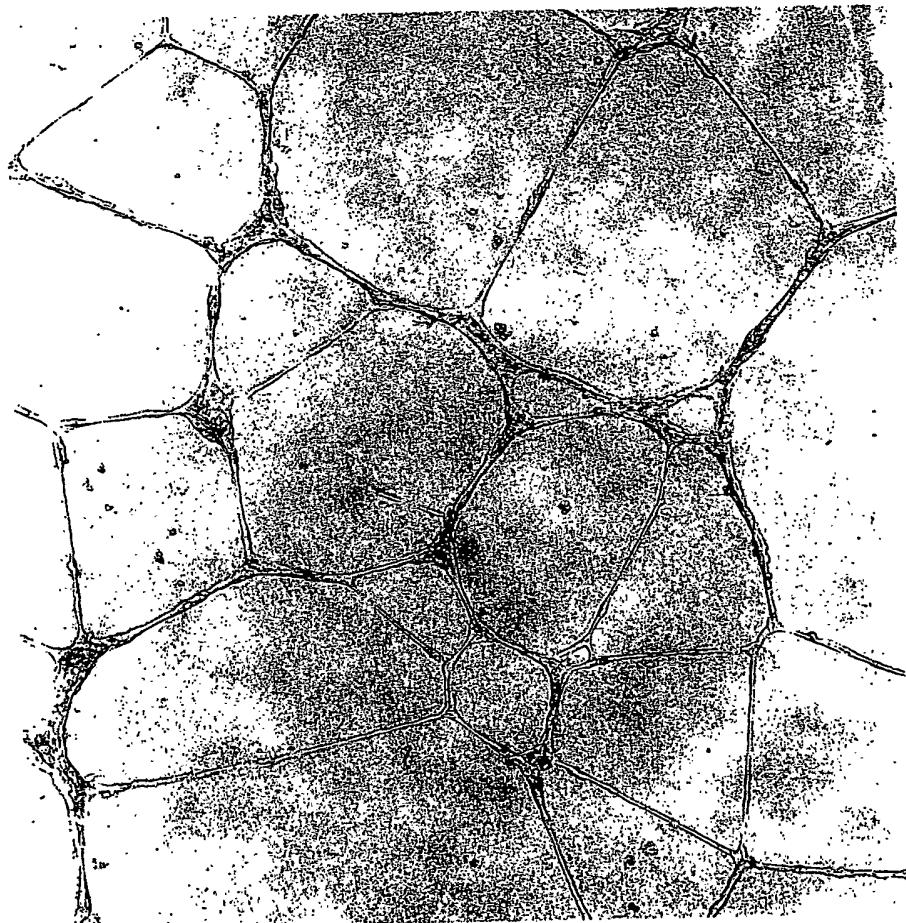
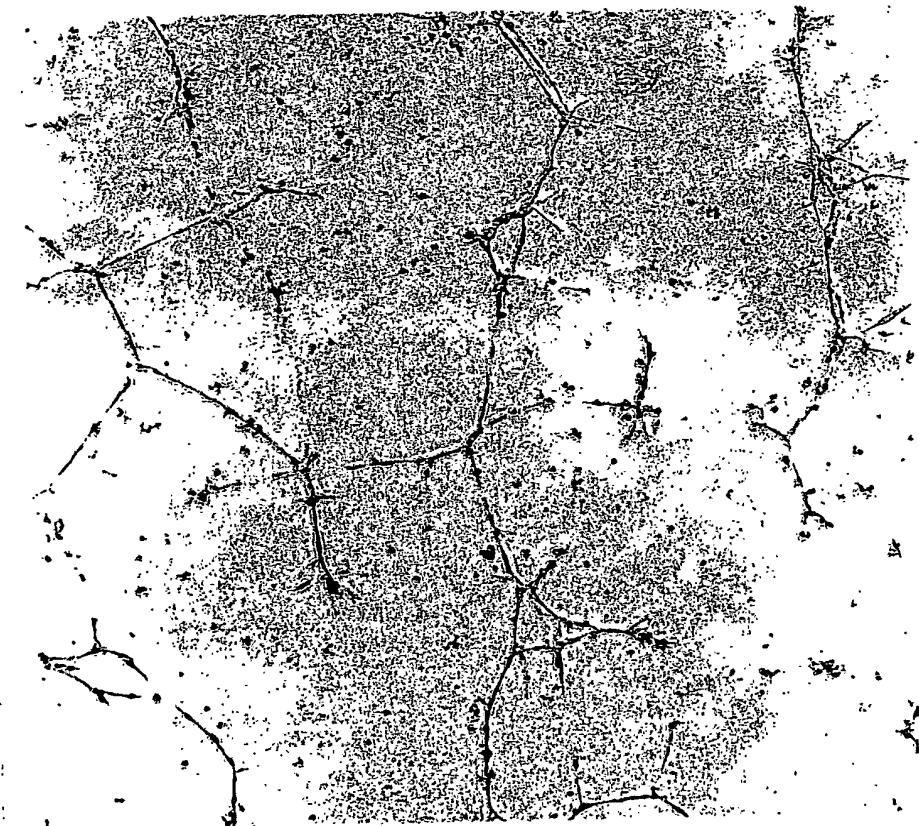


Figure 31: Endothelial microtubule formation by HUVECs on Matrigel (x 40 magnification). A visual analogue scale was used to determine the extent of tube formation and scored on a scale from 0 (all cells remain single) to 4 (all cells involved in tubular structures) as described in the text.

Dendrimer gen. 3.5-glucosamine 6-sulfate at 12.5 μ g/ml



Figures 32: Endothelial microtubule formation by HUVECs on Matrigel (x 40 magnification). A visual analogue scale was used to determine the extent of tube formation and scored on a scale from 0 (all cells remain single) to 4 (all cells involved in tubular structures) as described in the text.

Dendrimer gen 3.5-glucosamine 6-sulfate at 50 μ g/ml

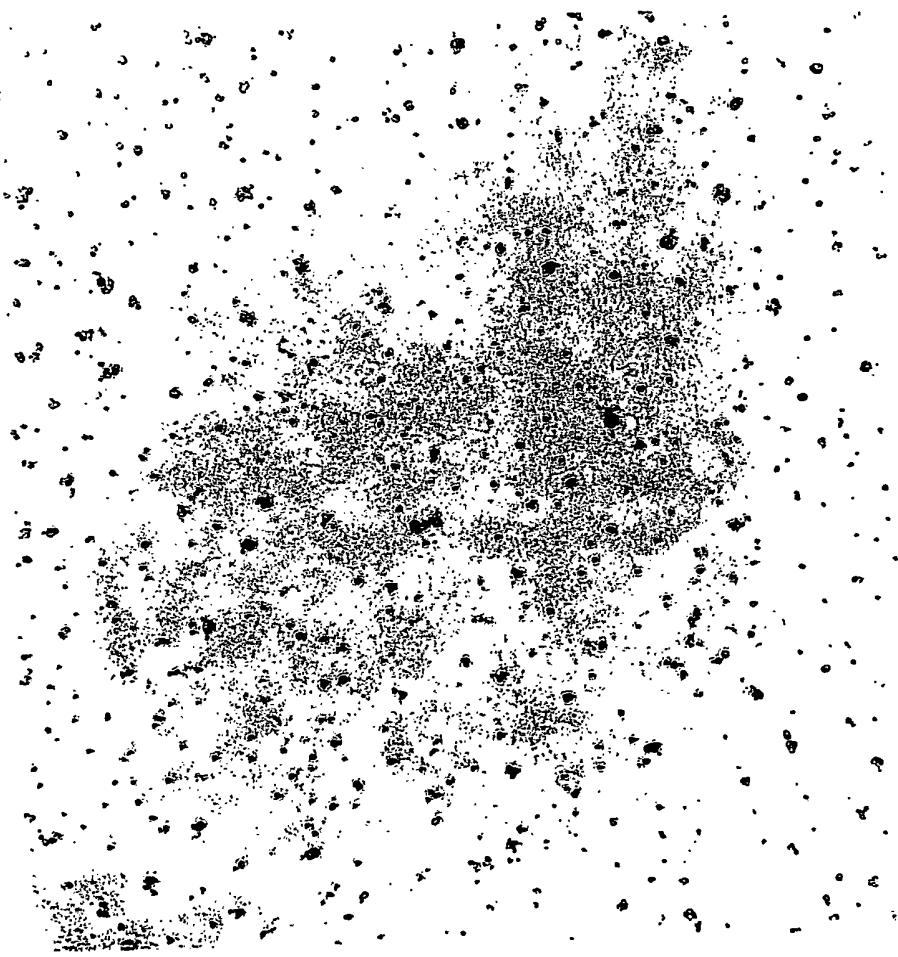
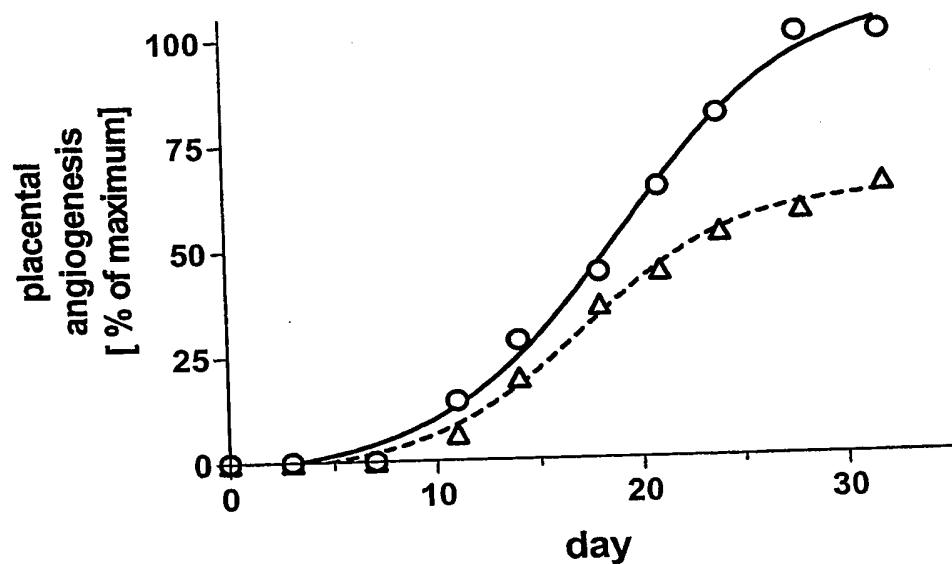


Figure 33: Dendrimer gen. 3.5 glucosamine 6-sulfate reduced the rate of new blood vessel formation in an *in vitro* human placental angiogenesis assay.



○ Control

△ Dendrimer gen. 3.5 glucosamine 6-sulfate (50 µg/ml)

New treatments for chronic inflammatory diseases

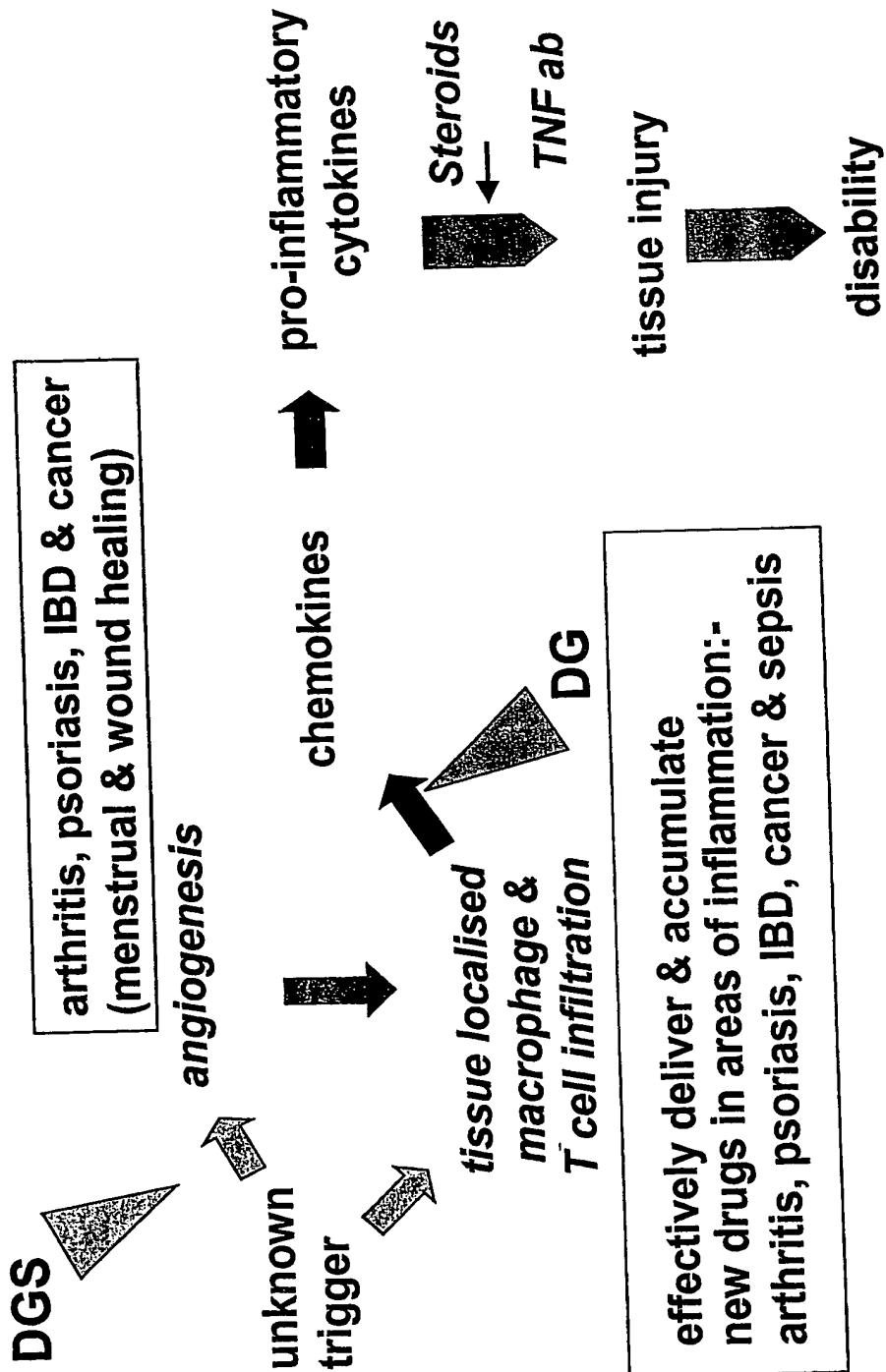


Figure 34

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